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PERSISTENT TIDAL CYCLES OF SPONTANEOUS MOTOR ACTIVITY IN THE FIDDLER CRAB, UCA PUGNAX [1]

MIRIAM F. BENNETT, JOAN SHRINER AND ROBERT A. BROWN

Sweet Briar College, Sweet Briar, Virginia, Northwestern University, Evanston, Illinois, and the Marine Biological Laboratory, Woods Hole, Massachusetts

Early in the present century, it was reported that a number of littoral organisms showed cycles of behavior which persisted under so-called constant laboratory conditions with tidal frequencies and with phases adaptively related to tidal events of the areas from which the organisms were collected (Gamble and Keeble, 1903, 1904; Bohn, 1904, 1906). Later, Gompel (1937) found that several species of animals display tidal rhythms of O_2-consumption which also persist under constant laboratory conditions. These reports were not very successful in convincing the majority of biologists of the reality of persistent tidal rhythmicity. However, during the past few years, a number of studies have again pointed out that many organic processes, *e.g.*, color change, spontaneous activity, and O_2-consumption, in a rather wide variety of plants and animals do indeed vary with primary lunar or tidal frequency under constant conditions.

Rao (1954) found that the filtering rate of species of *Mytilus* was greatest at the times of high tides in the areas of collection when the mussels were maintained in the laboratory. This rhythm of behavior was clearly apparent day by day. However, many of the lunar or tidal cycles that have been described are apparent only by statistical analyses of 15 or 29 days of continuous data (Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955; Brown, Shriner and Ralph, 1956).

The results of the work to be described demonstrate that the fiddler crab, *Uca pugnax*, does have an overt rhythm of primary lunar frequency, a rhythm of spontaneous motor activity.

Materials and Methods

In both 1955 and 1956, males of the species *Uca pugnax* were used in these studies. The crabs were collected from Chapoquoit beach or Sippiwisset beach on the Buzzards Bay side of Cape Cod. Tidal events on these two beaches occur roughly 10 minutes later than they do at New York City. In the laboratory the animals were kept in white-enamelled pans in a small amount of sea water until they

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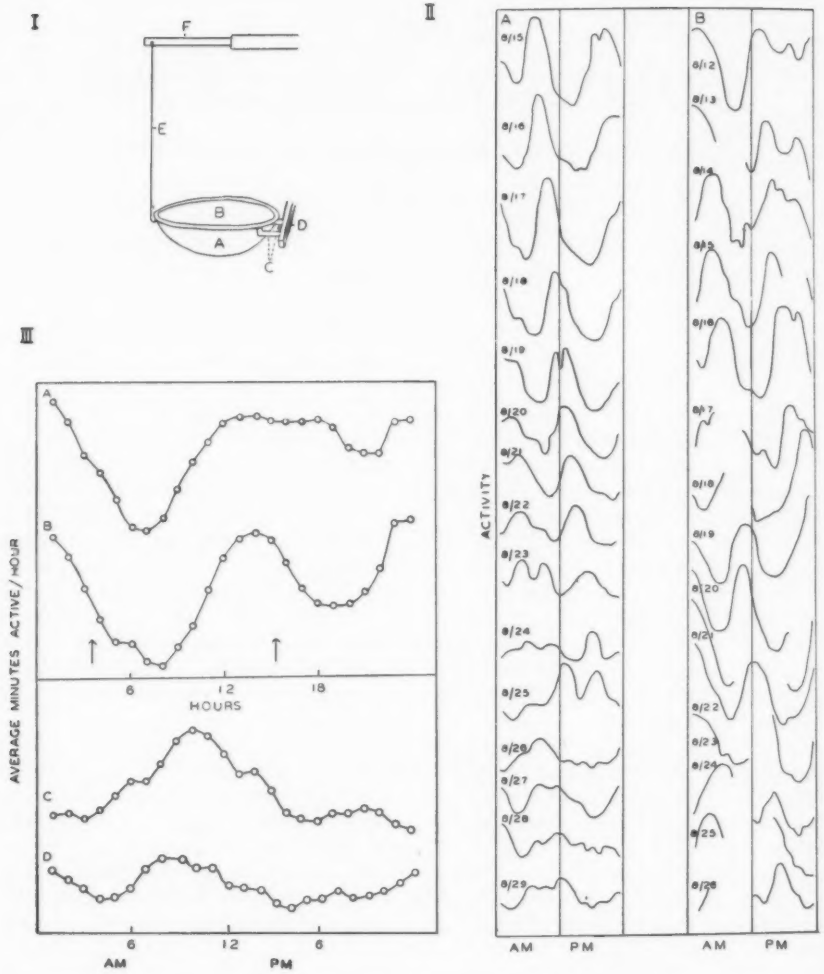

FIGURE 1. I. An illustration of part of the apparatus used to record the motor activity of an individual fiddler crab. For explanation of letters, see text. II. A, the average cycles of activity for a group of 20 crabs for the 15 consecutive days from August 15 through August 29, 1955. B, the average cycles of activity for a group of 20 crabs for the 15 consecutive days from August 12 through August 26, 1956. III. A and B, the mean, 29-day, tidal cycles of activity of fiddler crabs for July 6 through August 3, 1955 and August 2 through August 30, 1955, respectively. The arrows indicate the relative times of low tide at Chapoquoit Beach. C and D, the mean, 29-day, solar cycles of activity of fiddler crabs for July 6 through August 3, 1955 and August 2 through August 30, 1955, respectively.

were placed in the recording apparatus. This was done usually within two days of their collection.

Part of the recording apparatus employed is illustrated in Figure 1, I. A single crab was placed with a small amount of sea water in a plastic saucer (A), and covered with a circular piece of cardboard (B) which fitted the saucer tightly. Each of 10 saucers was supported on one side by metal bands (C) which were in turn fastened to a rigid horizontal bar (D). To the opposite side of each saucer was attached a nylon thread (E) which was fastened to the lever of a spring balance recording system (F) equipped with an ink-writing pen. The movements of the crabs in the finely balanced saucers were recorded on a kymograph which made one complete revolution every 24 hours. The spring balances and kymographs were of the type described by Brown (1954a).

The experiments were carried on in an inner room in a brick building at the Marine Biological Laboratory in which the light intensity at the level of the recording apparatus was at all times essentially constant and less than two ft. c. The containers with the inclosed crabs were shielded from movements and shadows in the laboratory. The air temperature in the room varied non-rhythmically from 21° to 24° C. through the summer months.

During the summer of 1955, the activity of 10 crabs was recorded continuously from July 6 through August 13, and that of 20 crabs was recorded from August 14 through 30. Freshly collected crabs were placed in the recorders on July 5, July 14, and August 14. In 1956, 20 crabs, which were collected on June 16 were placed in the recorders on that day, and their activity was recorded through the afternoon of June 26. On August 11, another group was collected, and the activity of 20 of these was recorded from that day through August 28.

The data recorded in 1955 were analyzed in the following manner: the number of minutes of each hour that each animal was active was determined from the kymograph recordings. From these figures was calculated the average number of minutes per hour that the population (either 10 or 20 individuals) was active. The hourly data were converted into three-hour moving means as given in Table I. Mean, 29-day solar and lunar cycles of activity were analyzed by methods used extensively in our laboratory (Brown, Bennett, Webb and Ralph, 1956; Brown, Freeland and Ralph, 1955) by which any possible solar or lunar cycles are synchronized day by day.

The results for 1956, given in Table II, are in terms of activity units per hour. Activity units were derived as follows: for each hour, the number of animals of the group of 10 that was active, *e.g.*, 8 out of 10, was recorded from the kymograph records. From these hourly values were calculated three-hour moving sums. The sums for the two groups of 10 each that were observed concurrently were added.

For the periods August 14–30, 1955, June 17–26, 1956, and August 12–28, 1956, when the motor activity of 20 individuals was recorded, the average values per hour for the two groups of 10 crabs each were correlated on an hour-by-hour basis. However, in this report, all data are given as the average for the entire population that was observed at any particular time.

Results

The hourly values of spontaneous motor activity for *Uca pugnax* in 1955 are found in Table I, and those for the two periods of 1956 in Table II. By merely

scanning these data, it is possible to observe that within many solar days there are two periods, each several hours in duration, of relatively high activity which are separated from one another by 12 to 13 hours, *e.g.*, on July 7, 1955 (Table I), there was high activity during the first two hours of the day and again during hours 12 and 13. By noting the situation on two consecutive days, *e.g.*, July 16 and 17, 1955 (Table I), it can be seen that the two periods of high activity occur later in the solar period on the second day than on the first. It is also evident from inspection of the data that there is a considerable range in the values, 0 minutes per hour active to 45

TABLE I

The average number of minutes active/hour for groups of fiddler crabs for 1955

		Hour																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
July	6	33	30	23	14	7	8	9	19	27	37	38	34	32	26	21	13	12	12	17	17	18	21	23	26
	7	29	29	22	19	10	11	8	16	23	32	34	39	39	33	25	17	16	—	—	—	24	17	13	11
	8	14	23	28	29	23	—	—	—	—	15	16	18	14	18	18	17	11	8	8	8	10	8	10	12
	9	17	20	16	17	12	10	6	4	3	3	6	10	17	20	14	9	6	6	5	4	8	9	8	7
	10	10	14	15	13	13	11	8	6	6	10	10	13	11	14	13	13	9	6	5	8	10	11	6	5
	11	6	9	12	14	13	10	9	7	7	6	6	4	4	7	11	10	8	5	6	5	6	6	8	5
	12	4	4	6	8	10	12	14	12	10	6	4	2	1	1	4	6	7	8	6	6	2	4	4	5
	13	3	3	3	7	10	10	7	5	—	—	—	13	10	7	5	1	6	11	13	8	4	3	3	4
	14	3	2	2	3	7	9	10	7	—	—	32	17	13	13	19	23	29	34	36	31	26	22	15	12
	15	12	8	10	24	36	45	41	36	28	21	15	9	6	3	7	13	20	22	28	30	32	28	24	20
	16	14	13	11	18	23	31	34	36	31	24	15	9	10	8	8	5	8	14	19	24	22	18	14	15
	17	15	9	6	6	10	18	22	23	23	20	16	13	8	7	6	7	7	5	7	11	14	15	13	14
	18	14	14	11	9	8	8	11	15	20	21	22	18	—	—	—	6	6	5	3	6	8	11	13	18
	19	17	15	8	6	8	8	8	7	13	15	19	14	12	5	2	1	1	4	4	5	3	5	9	11
	20	15	16	16	10	7	6	7	9	12	19	16	14	11	13	10	5	2	2	2	2	1	1	3	6
	21	7	5	5	6	12	12	12	8	5	3	5	7	—	—	—	9	5	3	2	2	4	4	5	4
	22	5	8	12	12	14	12	12	10	12	10	6	5	7	8	11	12	12	7	5	3	4	3	3	2
	23	4	6	9	15	17	19	12	9	7	8	9	6	4	4	4	4	5	5	3	1	3	6	9	7
	24	5	4	8	6	5	3	10	18	19	18	15	15	10	6	6	8	9	10	12	11	5	1	1	2
	25	5	6	6	5	3	4	3	11	23	31	26	17	8	8	7	7	7	7	7	9	9	12	9	8
	26	5	5	4	3	4	8	11	20	19	21	13	8	3	1	—	—	—	—	2	3	7	8	8	6
	27	6	6	3	2	2	5	10	11	11	9	11	10	7	5	2	3	2	2	3	3	3	5	7	7
	28	5	4	4	5	—	—	—	—	—	11	12	18	18	10	4	1	0	1	—	—	—	—	—	—
	29	—	—	—	—	—	—	—	—	—	—	—	24	21	16	11	8	5	3	2	3	6	11	15	11
	30	8	4	5	5	7	6	6	3	4	11	16	19	19	19	16	11	6	4	3	4	5	6	4	3
	31	3	4	7	12	15	18	16	16	14	13	15	19	21	23	19	14	9	8	9	9	7	7	8	11
Aug.	1	11	9	5	6	9	13	12	13	12	12	12	14	17	19	22	22	19	14	17	18	14	10	5	3
	2	3	5	6	7	6	6	6	10	11	8	3	5	12	19	22	20	20	20	18	16	11	10	6	7
	3	6	7	8	10	11	—	—	—	—	—	13	9	6	10	11	19	24	30	30	25	23	20	13	11
	4	12	12	12	11	14	15	14	12	7	10	12	12	9	7	10	8	10	11	11	11	9	12	10	11
	5	11	12	12	9	6	5	6	7	6	5	4	4	3	4	6	8	9	10	11	11	10	9	10	11
	6	10	9	5	5	4	6	7	7	8	8	8	4	3	4	5	4	3	3	5	6	6	7	9	9
	7	8	7	6	7	5	8	7	8	5	6	10	14	14	9	8	10	11	11	9	9	11	8	7	5
	8	9	8	7	5	7	10	8	8	7	8	9	8	7	5	3	5	7	8	7	6	9	11	11	10
	9	9	7	4	5	8	12	10	7	8	7	7	3	3	6	8	11	16	15	9	4	6	6	13	
	10	15	19	18	17	15	13	11	8	6	3	2	3	7	10	13	15	17	17	15	11	5	10	15	
	11	16	14	12	15	19	21	20	18	18	16	10	5	3	3	5	8	16	16	11	4	1	2	1	2
	12	7	11	11	9	11	16	18	19	19	17	12	7	7	6	9	9	11	14	16	16	15	13	11	7
	13	5	10	10	9	10	6	9	8	9	9	8	8	9	9	9	7	5	7	6	9	10	12	12	9
	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	19	17	21	23	20	25	26	26
	15	23	21	16	15	20	30	40	40	34	22	13	10	8	6	5	7	16	20	33	31	36	34	29	22
	16	17	15	13	11	14	19	35	40	37	26	19	15	14	13	10	11	10	16	19	25	29	31	31	31
	17	29	19	12	10	6	7	14	26	37	37	32	22	14	10	8	6	5	3	6	11	17	22	23	29
	18	25	19	14	13	12	7	7	9	17	27	31	27	24	17	11	7	5	4	4	6	12	18	21	28
	19	24	23	23	20	11	8	7	6	10	18	26	20	28	23	16	11	4	3	3	4	6	12	14	18
	20	19	20	20	16	14	12	11	10	6	13	16	24	24	23	18	14	10	8	6	4	5	6	13	16
	21	19	19	21	22	18	16	12	10	7	5	8	13	20	21	20	16	12	8	7	6	6	4	5	8
	22	10	14	17	17	16	13	11	11	9	7	7	10	14	19	20	17	12	7	6	5	5	4	6	7
	23	10	9	15	16	17	11	12	15	15	10	6	5	6	9	11	13	14	14	10	8	6	5	5	5
	24	8	10	10	11	13	12	11	10	12	12	11	8	7	6	6	7	11	17	15	7	7	7	9	8
	25	8	6	5	8	10	11	10	10	11	13	18	26	27	23	16	13	17	22	24	20	15	12	12	9
	26	10	11	14	16	16	18	20	22	20	17	15	13	13	13	12	13	12	11	12	13	13	13	16	20
	27	19	15	10	9	11	16	19	20	19	19	20	18	15	14	13	11	9	7	8	10	12	15	19	20
	28	21	16	12	9	9	11	14	13	14	16	17	17	15	13	13	13	11	12	10	12	12	12	10	7
	29	6	5	5	9	12	13	12	13	13	12	15	15	14	12	9	6	6	5	7	6	7	8	10	9
	30	10	10	10	8	7	6	7	7	8	9	11	13	14	14	11	9	6	7	4	—	—	—	—	—

TABLE II

The activity units/hour for groups of fiddler carbs for 1956

	Hour																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
June 17	20	28	36	41	37	30	27	23	22	18	18	17	18	24	30	37	35	33	30	29	26	24	25	28
18	26	24	29	36	41	37	31	26	23	22	19	17	17	18	17	24	30	37	35	30	25	24	28	29
19	28	26	25	31	38	44	42	—	—	—	—	—	26	20	16	13	15	20	21	23	21	23	23	24
20	26	27	29	30	32	34	36	36	33	30	25	19	17	19	22	20	16	17	21	30	32	32	31	28
21	24	18	19	21	21	20	25	31	33	31	28	26	20	16	11	12	10	13	19	26	29	30	28	28
22	27	26	25	22	24	23	24	28	31	33	32	33	28	20	14	17	23	23	22	24	30	30	26	25
23	27	29	27	26	29	28	25	24	30	42	42	40	30	25	20	18	16	12	9	13	22	23	30	27
24	26	25	24	21	24	24	24	21	24	32	33	31	28	23	18	11	10	9	10	8	19	26	35	30
25	28	25	27	27	27	24	24	21	21	25	29	30	28	25	17	12	7	11	14	12	11	14	26	34
26	33	30	29	27	27	26	23	19	15	20	23	27	27	26	20	16	11	—	—	—	—	—	—	—
Aug. 12	57	56	53	51	43	35	28	27	25	28	37	50	55	56	54	50	48	47	52	50	48	44	49	54
13	58	56	53	48	44	—	—	—	—	—	36	32	39	48	52	49	42	41	40	43	46	42	35	29
14	32	38	48	49	49	40	35	24	24	21	30	28	33	37	42	47	44	44	39	40	40	37	31	24
15	28	37	46	—	—	—	36	29	28	24	18	18	22	32	41	47	45	38	—	—	—	—	38	32
16	24	20	24	32	37	40	35	27	16	13	13	11	9	9	15	29	42	44	43	39	43	36	26	20
17	17	26	24	29	29	—	—	—	—	22	17	13	13	14	10	7	14	24	30	31	27	26	22	21
18	15	13	13	17	21	25	—	—	—	—	—	13	9	11	11	13	12	14	18	27	35	42	40	34
19	24	20	16	14	8	8	10	20	28	30	31	26	20	14	11	10	10	12	17	20	25	34	41	40
20	34	27	22	16	14	14	16	25	34	40	36	29	20	14	8	7	6	12	14	—	—	—	44	41
21	39	35	29	24	17	13	9	10	—	—	—	—	—	—	—	—	14	9	9	7	7	11	23	35
22	38	34	26	23	19	16	10	10	15	23	28	32	31	29	23	16	11	8	10	10	11	16	23	30
23	36	35	34	27	24	20	21	17	17	17	19	—	—	—	—	39	21	15	13	12	10	11	13	28
24	19	24	28	32	36	36	36	31	—	—	—	15	14	20	22	26	20	15	11	11	9	12	12	17
25	21	28	33	33	29	25	—	—	—	—	13	12	—	—	—	32	30	22	18	13	13	11	11	11
26	16	20	23	—	—	—	—	—	—	—	—	22	22	19	23	29	34	33	28	23	21	17	17	19
27	25	25	27	26	31	32	38	37	32	30	28	25	19	16	22	23	25	25	27	27	26	23	20	17
28	16	18	21	20	25	27	32	32	33	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—

minutes per hour active in 1955, and 8 activity units per hour to 58 activity units per hour in 1956. Near the end of a particular recording period, both the mean daily activity and the amplitude of the cycles are lower than at the beginning of the same period.

These results are shown graphically in Figure 1, II A and B, in which are plotted the cycles of motor activity for groups of 20 crabs for August 15 through 29, 1955 and August 12 through 26, 1956. In Figure 1, II A, one can follow the moving of the two peaks of activity across succeeding solar days from August 15 to 22. In this instance, the peak that occurred between hours 7 and 8 on August 15 occurs between hours 14 and 15 on the 22nd, and the evening peak of August 15 has moved to the early morning on August 22. For the cycles of August 23 through 29, it is more difficult to distinguish maxima or the movement of these maxima very precisely. There seems to be a warping and diminution of a peak as it moves into the afternoon and evening hours. The peak of the morning hours which can be identified on August 20, can be traced through August 29; however, it, too, shows warping and broadening.

Generally, the same characteristics are to be seen in Figure 1, II B, especially for the cycles of August 12 through 16. After this time, the data are incomplete, and it is not advisable to complete the curves from only the available data. A period of high activity seen just after 0 hour on August 12 is identified as a rather sharp maximum at 12 on August 22, and similarly the afternoon high (hour 14) on August 12 is identified between hours 3 and 4 on August 25. Again, the warping and broadening of maxima, observed in 1955, are evident.

In order to demonstrate the reality of the movement of peaks of activity across solar days at the average primary lunar or tidal rate of 51 minutes per day, the rate of movement for specific peaks was determined for 20 different intervals from the data of 1955 and 1956. The intervals used were in no case less than two days or more than 11 days. The average rate for these periods was 50.1 ± 3.58 minutes per day.

Tidal phase relationships as well as frequency relationships are to be noted in these rhythms of activity. It was observed that in 1955, the peaks of activity occurred two to three hours before the times of low tide on the beaches from which the crabs were collected. In 1956, the maximum activity was, on the average, four to five hours before low tides.

This phase relationship is shown in Figure 1, III in which are plotted the mean, 29-day tidal cycles of locomotor activity for July 6 through August 3, 1955 (A) and August 2 through 30, 1955 (B). These curves are plotted so that the times of low tides at Chapoquoit Beach, as indicated by arrows, lie directly under one another, although the tidal events actually occurred at different times on the two days with which the 29-day analyses were begun. The times of low tide were: 3:22 and 15:19 on July 6 and 1:34 and 13:37 on August 2. In these figures, the form of the tidal cycle, discussed previously, is again apparent. In A, a sharp peak of activity is seen at hour 1, or 2 hours and 22 minutes before low tide. The second maximum takes the form of a broad peak from hour 13 through 18, extending from 2 hours before until 3 hours after low tide. Activity fell rather steeply from 1 until 7 while the activity following the second maximum did not decrease so steeply nor to so great a degree. The cycle for August 1955 (Fig. 1, III B) was very much like that illustrated in Figure 1, III A, although the second period of high activity does not last so long in the former as in the latter. Again, the activity persisted at a higher level following the second maximum than it did following the first peak.

In Figure 1, III C and D are plotted the mean, 29-day, solar cycles for the same periods of time as the lunar cycles. It is apparent that the amplitude of the solar cycles is less than that of the lunar cycles. From the lowest to the highest values there was a 2.4-fold increase in the tidal cycle for July 6 through August 3, and a 2.2-fold increase in the tidal cycle for August 2 through 30, whereas the increases for the solar cycles for the same periods were 1.6-fold and 1.3-fold, respectively. Although the amplitude of these solar cycles is low, the cycles indicate that activity between hours 6 and 12, other factors equal, is higher than at other times of the solar day. This tendency can be seen in Figure 1, II A by comparing the heights of the two peaks, *i.e.*, the morning peak is higher than the afternoon peak from August 15 through 17.

Coefficients of correlation for the activity of two groups of crabs, the activities of which were recorded independently during the same periods of time, were: + 0.768 ± 0.028 for August 14 through 30, 1955; + 0.410 ± 0.058 for June 17 through 26, 1956; and + 0.687 ± 0.034 for August 12 through 28, 1956.

Discussion

The cycle of spontaneous motor activity for the fiddler crab, *Uca pugnax*, described in this report, appears to be the first example of a clearly overt locomotor rhythm of primary lunar or tidal frequency. The occurrence of two peaks of ac-

tivity, 12 to 13 hours apart, within one solar day, and the movement of these peaks across succeeding solar days at an average tidal rate establish the reality of a tidal cycle persisting under laboratory conditions. It must be pointed out that the day-to-day preciseness of this rhythm decreases somewhat after the crabs have been in the recording containers seven or eight days. The warping and broadening of peaks, as well as the diminution of activity, discussed previously, cannot be explained satisfactorily at the present time. It is tempting to postulate that these phenomena are indications that an internal timing mechanism is not able to maintain a precise cycle longer than a week or so under constant conditions, and that after this time unknown external signals alone maintain only a less regular rhythm. Evidences for both endogenous and exogenous components of persistent rhythmicity in fiddler crabs have been reported recently (Brown, Webb and Bennett, 1955; Brown, Webb, Bennett and Sandeen, 1955).

The difference between the phase relationships of actual tidal events and the peaks of activity observed in 1955 and 1956 brings up questions regarding the setting of phases of tidal cycles, and to what extent behavior cycles under constant conditions parallel those of the organisms in their natural environments. The lunar or tidal cycles described previously indicate that although many species have cycles of 12.4 and/or 24.8 hours, phase relationships are species specific. For example, the spontaneous activity of quahogs is low during the times of low tides when this species may not be covered by water (Bennett, 1954), while the pigment in the melanophores of the fiddler crab, *Uca pugnax*, is most dispersed shortly before the times of low tide when these animals are typically active on the beaches (Brown, Fingerman, Sandeen and Webb, 1953). In these cases, the phases of the persistent tidal cycles seem to indicate some adaptiveness of the cycles to conditions which obtain under natural field conditions. On the other hand, Fingerman (1956) has reported that fiddler crabs, *Uca pugilator* and *Uca speciosa*, collected from regions of the Gulf coast where there is but one low tide per day, have persistent tidal cycles of color change characterized by two peaks of pigment dispersion during each solar day, one shortly after low tide, and the second 12.4 hours later or shortly after high tide.

The relationship observed between the fiddler crab activity and the times of low tide in 1955, *i.e.*, maximum activity two to three hours before low tides, might suggest that the behavior of the crabs in the laboratory is much the same as that of the crabs on the beaches. Typically, these crabs begin to emerge from their burrows as the water recedes following a high tide, and great numbers of these crabs are usually running on the beaches until shortly before low tide. However, the observations for 1956, *i.e.*, that the peaks of activity occurred four to five hours before low tide, suggest that the phase relationships of persistent tidal cycles change, and may not always reflect adaptive behavior of the species in its natural, changing, physical environment. It is possible that the difference in the rhythmic behavior of the crabs between the two years may reflect differences noted in field observations between these same two years. In 1955, as was usual, the crabs were collected easily shortly before the times of low tides. In 1956, the animals were difficult to collect since there were few running on the beaches before low tides. Many of the crabs used in 1956 had to be dug from their burrows. It is possible that stimuli other than those resulting from emergence and running of the crabs may set the phases of the observed tidal cycles.

That the phases of a tidal cycle do shift in the absence of experimental modifications is shown clearly in the report of Brown (1954b). In this work, it was found that the maxima of the tidal cycle of oysters collected from New Haven Harbor and shipped to Evanston, Illinois correlated with the times of high tide in the native habitat during the first two weeks of maintenance under constant conditions. During the next month, the same group of oysters showed a rhythm with maxima at the times of lunar zenith and nadir. Two other reports contain information regarding the experimental shifting of the phases of tidal cycles (Brown, Fingerman, Sandeen and Webb, 1953; Rao, 1954). However, since the problem of the setting of phases of persistent tidal cycles is one that has not been investigated to a great degree as yet, much more work must be done before any definite statements can be made.

The overt tidal rhythm described here promises to be one by which not only the problem of phase setting but also experimental modifications of tidal cycles can be studied. The facts 1) that the cycle of locomotor activity repeats itself rather precisely on a day-to-day basis for at least a week after the crabs are placed under constant conditions, and 2) that the cycles of two independent small groups correlate significantly to a high degree do point to its usefulness in such studies.

Summary and Conclusions

1. The spontaneous locomotor activity of groups of fiddler crabs, *Uca pugnax*, was recorded during the summers of 1955 and 1956 under constant laboratory conditions.

2. This species shows an overt rhythm of activity of primary lunar or tidal frequency. Within solar days, there are two peaks of activity which are 12 to 13 hours apart. These maxima move across succeeding solar days at an average tidal rate. The cycles are precise for at least a week under constant conditions, but after this time, some warping and displacement of maxima occur.

3. A low amplitude solar rhythm of activity is apparent upon analysis of 29 days of continuous data. This rhythm is characterized by high activity between hours 6 and 12 of the solar day.

4. There was observed a difference in phase relationships of the tidal rhythms between 1955 and 1956. The state of the problem of the setting of phases of persistent tidal cycles is discussed.

5. Since this rhythm is precise for at least a week under constant conditions, and since the cycles of two groups of crabs recorded independently correlate to a high degree, this cycle appears to be an excellent one with which to study experimental modifications of persistent tidal rhythms.

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STUDIES OF THE METABOLISM OF PHOSPHORUS IN THE DEVELOPMENT OF THE SEA URCHIN, STRONGYLOCENTROTUS PURPURATUS[1]

ALBERT L. BOLST[2] AND ARTHUR H. WHITELEY

Department of Zoology and the Friday Harbor Laboratories, University of Washington, Seattle 5, Washington

The eggs of sea urchins contain a group of acid-soluble phosphorylated compounds whose barium salts are soluble in alcohol. The magnitude of this fraction is unusually large in comparison with other animal tissues. The unfertilized eggs of various sea urchins and asteroids have been reported to have from 9.3% (Mende and Chambers, 1953) to 39.7% (Whiteley, 1949) of their acid-soluble phosphorus in the form of barium-soluble, alcohol-soluble compounds. In various vertebrate tissues these compounds comprise about 1 to 8.3% of the acid-soluble phosphorus (LePage, 1948; Sacks, 1949). Aside from quantitative measurements, very little is known about these compounds or the part they play in the metabolism of the echinoderm egg. Lindberg (1943) reported finding a compound in this fraction from the egg of the heart urchin, *Brissopsis*, which he subsequently (1946) identified as 1, 2-propanediol phosphate, whose metabolism he studied. Hörstadius and Gustafson (1947) reported some animalizing effect by both synthetic propanediol phosphate and a natural compound, and Borei (1948) has described the effect of this ester on egg respiration. However, Rudney (1952, 1954) has reported that the barium salt of propanediol phosphate is not soluble in alcohol, and the significance of the above observations, therefore, is not clear. Mende and Chambers (1953) have shown that some of the phosphorus of the barium-soluble, alcohol-soluble fraction of *Asterias forbesii* and *Strongylocentrotus dröbachiensis* is acid-labile.

This paper supplies information relative to the extent to which the barium-soluble, alcohol-soluble fraction serves as a storage material for metabolism, and the rate of turnover of the compounds of this fraction in the eggs and embryos of the sea urchin, *Strongylocentrotus purpuratus*. The possibility has been explored that there are special periods in the developmental process when the metabolic activity of these compounds varies. The existence of propanediol phosphate as a component of this fraction has been examined, and preliminary chromatographic determination of the number of esters in the fraction has been made. As an outgrowth of the turnover studies, an unusual pattern of permeability of embryos to inorganic phosphate during larval development has been found.

[1] This investigation was supported in part by the Research Fund 171 of the University of Washington. A report of these studies was given at the meeting of the Western Society of Naturalists, December, 1952.

[2] Currently on duty in the Navy.

Materials and Methods

Embryological. The sea urchins used in these experiments were *Strongylocentrotus purpuratus* (Stimpson), collected from San Juan Island, Washington. Animals were induced to spawn by the injection of isotonic KCl (Tyler, 1949). Batches of eggs less than 95% fertilizable were not used. "Dry" sperm were diluted to a 1% suspension immediately before use. After insemination, excess sperm were removed from eggs by washing with fresh sea water. Filtered sea water was used throughout.

In experiments on embryos older than the two-cell stage suspensions of developing embryos were cultured in a four-liter Erlenmeyer flask which lay at an angle of about 30° in a water bath held to 11.0 ± 0.1° C. by a thermostat. Egg suspensions, ranging in concentration from 0.3 to 0.9% by volume, were gently agitated by rotating the flask at 28 rpm. For egg counts, 10-ml. aliquots were taken and, after appropriate dilution, ten individual counts were made and averaged.

Aliquots of 300 ml. were taken from the stock suspensions at different stages of development for analysis of phosphates and for separate incubation with radioactive phosphate. Carrier-free radioactive phosphate was added to give a final radioactivity of 0.04 μc./ml., and incubation was continued in the same manner as described above, normally for 60 minutes. Duplicate 10-ml. aliquots were then taken for analysis of total phosphorus. The embryos were removed from the remainder of the suspension in a lucite centrifuge patterned after the Foerst plankton centrifuge. After washing with fresh sea water the embryos were frozen and stored for later analysis.

In experiments with eggs prior to the first cleavage the incubation times and radiophosphate concentrations varied and are given with the results. These samples were analyzed immediately without freezing.

Analytical. The extraction of the barium-soluble, alcohol-soluble material from the embryos followed the procedure of Sacks (1949) and that of Umbreit, Burris and Stauffer (1949). In some instances the other phosphate fractions identified below were also prepared. The sample was thawed and homogenized in a Potter-Elvehjem type of homogenizer in 2 ml. of 0.5 N $HClO_4$. This and the subsequent steps were carried out near 0° C. The homogenate was centrifuged and the residue (acid-insoluble fraction) re-homogenized twice with 1 ml. of 0.5 N $HClO_4$. The three supernatants were pooled, four volumes of re-distilled 95% ethyl alcohol added, and the mixture set aside for one hour. The extract was centrifuged, the residue (acid-soluble, alcohol-insoluble, probably polysaccharides) washed twice with acid-ethanol, the combined supernatants adjusted to pH 8.2, and 1 ml. of 25% barium acetate added. The precipitate (barium-insoluble plus barium-soluble, alcohol-insoluble fractions) that formed after one hour was centrifuged and washed with ethanol adjusted to pH 8.2. The supernatants were brought to 50.0 ml. This is the barium-soluble, alcohol-soluble fraction.

Phosphorus was determined by the method of Berenblum and Chain (1938), and, in a few cases, by a modified Fiske and SubbaRow (1925) method with ferrous sulfate as reducing agent. The dried samples, blanks, and standards were digested in 70% $HClO_4$. The blue isobutanol extracts resulting from the phosphorus determinations were used for radioactivity assay. Aliquots were pipetted into planchets, dried, and counted with a Geiger-Müller counter equipped with an end-window tube

with window thickness of 3.26 mg./cm.^{2} Samples were counted to an error of less than 1%. In experiments with pre-cleavage stages, where phosphate was analyzed by the Fiske and SubbaRow method, aliquots of eggs or extracts were dried directly on planchets.

Paper chromatographic analysis for phosphate esters was carried out using a chromatographic system the details of which will be published elsewhere. The solvent system contained n-butanol, n-heptyl amine, and water. The descending method was used with washed Whatman No. 1 filter paper. The chromatograms were run at 1° C. for about 10 hours and developed by spraying with the Hanes-Isherwood molybdate reagent, heating at 80° C. for 5 minutes (Hanes and Isherwood, 1949), and irradiating with ultraviolet light at wave-length 2537 Å (Bandurski and Axelrod, 1951).

Results

If the phosphorus-containing compounds of the barium-soluble, alcohol-soluble fraction serve as a reserve of phosphorus, energy, or precursors of other substances during development, an indication of this function would probably be given by a decrease in the concentration of the phosphorus in the fraction as development progresses. Two experiments, each involving the eggs of a single sea urchin, were carried out to determine if this occurs. In each a large batch of fertilized eggs was cultured at 11° C. to the early pluteus stage. Aliquots were taken at intervals for determination of total phosphorus and barium-soluble, alcohol-soluble phosphorus. The results are given in columns three and four of Table I, and are shown in

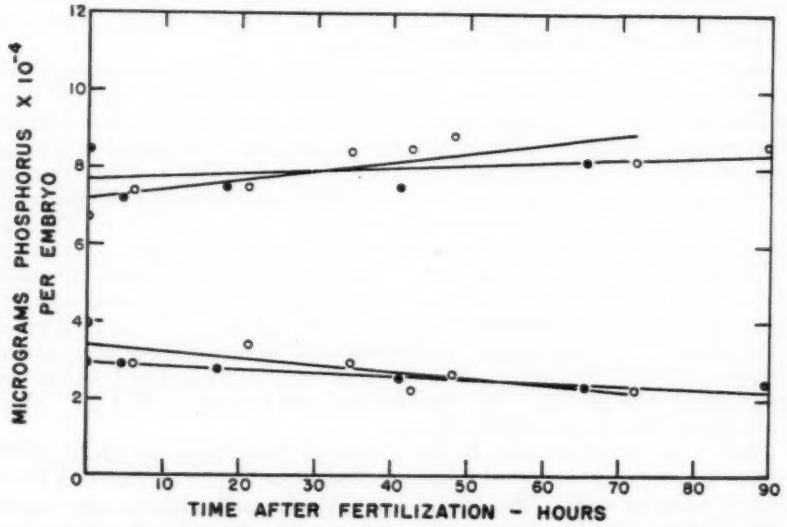

Figure 1. Total (upper curve) and barium-soluble, alcohol-soluble phosphorus content (lower curve) of developing embryos of *Strongylocentrotus purpuratus*. Solid circles are data from Experiment 1 and open circles are from Experiment 2 of Table I.

TABLE I

Amount of phosphorus and rates of incorporation of radioactive phosphorus in embryos and barium-soluble, alcohol-soluble compounds of embryos of the sea urchin, Strongylocentrotus purpuratus

Embryos		Phosphorus content μg./egg		Radioactive phosphorus content (cts./min./egg/hr.)		% BSAS-P_{32}	Specific activity (cts./min./μgP/hr.)		% BSAS-P_{32}
Age	Stage	BSAS-P	Total P	BSAS-P_{32}	Total P_{32}		BSAS-P_{32}	Total P_{32}	
Experiment No. 1									
0 hrs.	UF*	3.0×10^{-4}	8.5×10^{-4}	—	—	—	—	—	—
4½	2-Cl	2.9	7.2	6.5×10^{-2}	120×10^{-2}	5.4	225	1710	13.2
17	UB	2.8	7.6	15.2	221	6.9	532	2910	18.3
41	EG	2.5	7.6	2.8**	36**	7.8	108**	472**	22.9
65½	LG	2.3	6.2	13.8	193	7.2	595	2240	26.6
89½	EP	2.4	8.6	6.4	140	4.6	262	1550	16.9
Experiment No. 2									
0	UF	3.9	6.7	—	—	—	—	—	—
6	4-Cl	2.9	7.4	3.9	56	7.0	134	732	18.3
21	UB	3.4	7.6	13.3	241	5.4	384	3090	12.4
34½	EG	2.9	8.4	24.8	392	6.4	863	4520	19.1
42½	EG	2.2	8.5	19.0	332	5.7	868	3760	23.1
48	MG	2.6	8.8	17.3	248	7.0	657	3360	19.6
72	PR	2.2	8.2	7.2	141	5.1	324	1670	19.4
Experiment No. 3									
43	EG	3.0	12.0	29.6	559	5.3	1120	5420	20.6

* Eggs from a single female used for each experiment. UF = unfertilized, 2-Cl = 2-cell stage, 4-Cl = 4-cell stage, UB = unhatched blastula, EG = early gastrula, MG = mid-gastrula, LG = late gastrula, PR = prism, EP = early pluteus.

** These data low, presumably by a factor of 10, due to a technical error. Experiment No. 3 was run at 43 hours to check this point.

Figure 1, the curves of which are fitted by the method of least squares. The quantity of barium-soluble, alcohol-soluble phosphorus decreases at a uniform rate throughout the non-feeding stages of larval development. The rate of utilization in Experiment 1 is 7.4×10^{-7} microgram/egg/hour and in Experiment 2 is 20×10^{-7} microgram/egg/hour. The per cent of the fraction present at the beginning of development utilized in reaching the prism stage (72 hours) is 18.5% and 40.7% for Experiments 1 and 2, respectively (based on the fitted curves). It seems safe to conclude that the utilization of the material contributed appreciably to the general metabolism since it involves mobilization on the average of 13.6% of the total phosphorus of the unfertilized egg. No significant variations in the rate of utilization correlated with visible aspects of morphogenesis are apparent in either experiment.

TABLE II

Distribution of radiophosphorus in barium-soluble, alcohol-soluble and other phosphorus-containing fractions in unfertilized and fertilized eggs. The per cent of radiophosphorus in each fraction is calculated with acid-insoluble plus acid-soluble equal to 100%

Fraction	Unfertilized eggs*			Fertilized eggs**		
	cts./min./ml.	Per cent P^{32}	Specific activity cts./min./μg P	cts./min./ml.	Per cent P^{32}	Specific activity cts./min./μg P
Total egg	—	—	—	188,500	100.3%	240
Acid-insoluble	24,050	29.2%	28.3	14,690	7.87	33.4
Acid-soluble	58,600	71.0	75.1	172,100	92.0	486
Barium-insoluble plus barium-soluble, alcohol-insoluble	60,100	73.0	95.5	160,500	85.9	596
Barium-soluble, alcohol-soluble	1,180	1.40	4.37	1,350	0.72	11.6

* Incubated 9 hours in P^{32} concentration of 0.04 μc/ml.
** Incubated one hour, before first cleavage, in P^{32} concentration of 0.02 μc/ml.

Among analyses done by both of the present authors, as well as those by others, the variation in the quantity of the barium-soluble, alcohol-soluble phosphorus is broad. Data of the present authors include values of 3.9, 3.0, 1.05 and 0.98×10^{-4} micrograms per egg, and many other analyses for which egg counts are not available indicate that the fraction from unfertilized eggs contains from 12.0% to 58% of the total phosphorus. Variations in multiplicate analyses rarely are of appreciable magnitude, usually amounting to only a few per cent; it is believed the large differences from batch to batch represent true biological variations. Sacks (1949) reported variations of comparable magnitude in this fraction extracted from rat liver. Mende and Chambers (1953) found different batches of eggs of *Asterias forbesii* to contain 322, 153, and 138 μg. P/ml., and *Strongylocentrotus dröbachiensis* to contain 59 and 44 μg. P/ml.

During the period of development covered in these experiments there was an increase in the total egg phosphorus. In Experiment 1 this amounted to an increase of 9.1%, and in Experiment 2 of 23%, as calculated from the fitted curves.

Although the barium-soluble, alcohol-soluble fraction shows an appreciable decrease during development, the possibility exists that there is a simultaneous synthesis of some of the compounds in the fraction. The question of synthesis was examined in unfertilized eggs and in embryos by adding radioactive inorganic phosphate to cultures and determining the radioactivity of the fraction and of the whole eggs after appropriate time intervals.

Unfertilized sea urchin eggs take up radioactive phosphate from sea water at an extremely low rate. To obtain sufficient activity in the phosphate fractions of such eggs, they were incubated for 9 hours at 12.0° C. in sea water containing 0.04 μc. radiophosphate/ml. The egg concentration was 0.4% by volume. Of a small sample inseminated after this incubation, more than 95% fertilized and developed to the early pluteus. The data in Table II show that the incorporation into the barium-

soluble, alcohol-soluble fraction prior to fertilization is very low; even after prolonged exposure to radiophosphate only 1.4% of the total activity of the egg was in this fraction. In a second experiment the figure was 0.78%. In confirmation of other investigators most of the activity enters the fraction containing inorganic phosphate, nucleotide phosphate, and labile esters, though in the 9-hour experiment very much more is found in the acid-insoluble fraction than has been reported before (Chambers and White, 1954).

The same low rate of incorporation of phosphate into this fraction persists directly after fertilization, before the first cleavage. This is apparent from the second experiment of Table II, in which a 1% suspension of eggs inseminated 12 minutes earlier was incubated for 60 minutes with 0.02 μc./ml. of radiophosphate. Although considerably more phosphate penetrated into these than into unfertilized eggs, the proportion in the barium-soluble, alcohol-soluble fraction is still only 0.7% of the whole. In comparable experiments, neither mono-iodoacetate nor 2,4-dinitrophenol, added with the radiophosphate, changed this pattern markedly, though both inhibited the uptake of phosphate by the egg.

The subsequent embryonic period was examined from the two-celled embryo to the early pluteus stage at 90 hours in the experiments of Table I. In these the time of exposure to radiophosphate at each stage examined was 60 minutes. Columns 5 through 10 of Table I present the pertinent determinations of radioactivities. Of the radiophosphorus that enters the embryos during one hour, an average of 6.5% (4.6 to 7.8%, Column 7) is incorporated into the fraction. There is no consistent pattern of change in this value during the rest of the development. Considered in terms of specific activities, the fraction attains a level that is about 19% (12.4% to 26.6%, Column 10) of the specific activity of the total egg phosphorus, again with no consistent change during the rest of the development. This contrasts with 5% for the freshly fertilized eggs in the experiment of Table II. Although the components of this fraction are metabolically very inactive in the unfertilized egg and before the first cleavage, it is concluded that one or two hours after fertilization at least part of the barium-soluble, alcohol-soluble fraction becomes moderately stimulated metabolically relative to phosphorus compounds of the egg as a whole, and this increased level of metabolic activity is maintained with approximate constancy until the pluteus stage.

The specific activities of the total egg phosphorus and of the barium-soluble, alcohol-soluble fraction are plotted against age of the embryos in Figure 2. It should be noted that these curves are not cumulative uptake curves, but rather are rate curves, each point representing the counts per minute per microgram of phosphorus per 60 minutes exposure at the particular age indicated. The rate of incorporation into the fraction is seen to increase to a maximum at about 40 hours, and then decline subsequent to this time. The peak of activity at 40 hours probably does not represent a special stimulation in metabolism within the fraction leading up to gastrulation, because, as was pointed out above, the activity of the fraction, when expressed as percentage of the whole embryo, is constant. Rather the peak reflects closely the uptake curve for the whole embryo, and is probably due to a change in permeability of the embryo to inorganic phosphate from the sea water.

An unanticipated finding in these experiments is that the rate of uptake in both the total phosphorus and the barium-soluble, alcohol-soluble fraction varies markedly in the different phases of development. The rate of uptake which begins to increase

15 or 20 minutes after fertilization (Abelson, 1947; Brooks and Chambers, 1948; Whiteley, 1949) continues to increase markedly until the stage of early gastrulation, at 34 to 43 hours in the different experiments. The rate then begins to decrease

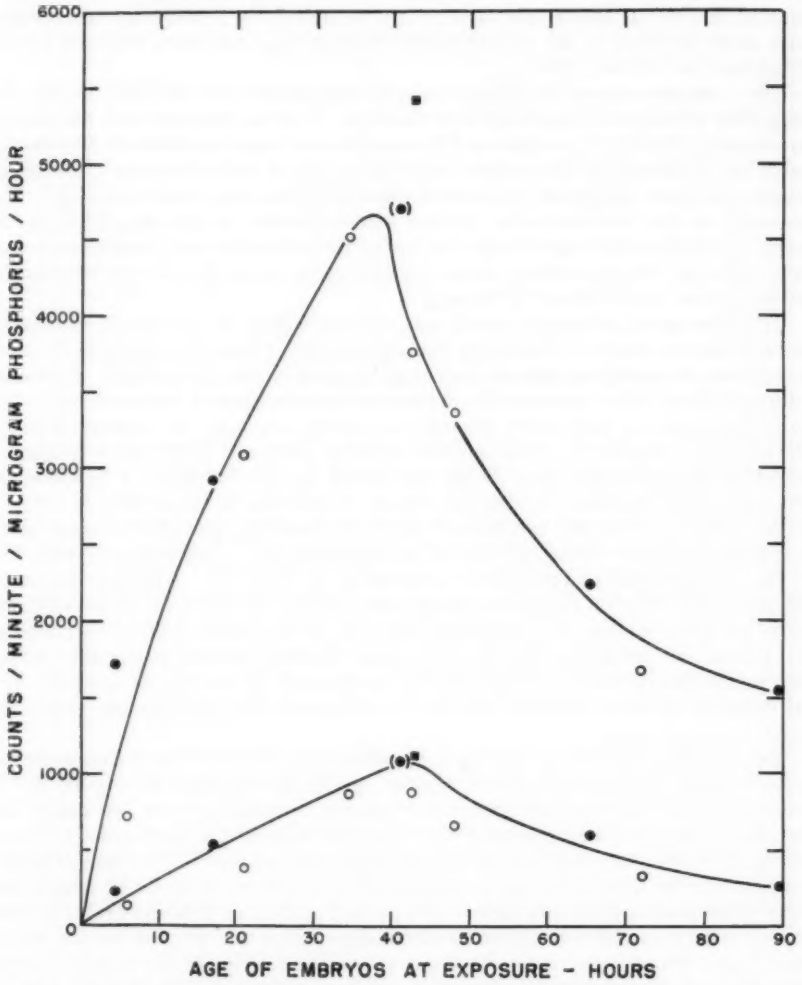

FIGURE 2. Rate of uptake of radiophosphate into the embryos of *Strongylocentrotus purpuratus* (upper curve) and into the barium-soluble, alcohol-soluble phosphate fraction (lower curve) of these embryos. Solid circles are data from Experiment 1, open circles are from Experiment 2, and squares from Experiment 3 of Table I. The bracketed solid circles at 41 hours have been multiplied by 10 (see footnote to Table I). Specific activities are in counts/min./μg. P/hour.

sharply, tending to level off in the prism and early pluteus stages. As nearly as could be determined, the inflection in the rate coincides with the onset of gastrulation. This pattern of uptake is shown when the data are considered either on the basis of counts per minute per egg or of specific activity.

This change of permeability to orthophosphate is not, however, universal for all phosphate compounds. The permeability of embryos to propanediol phosphate, determined in an experiment similar to those with orthophosphate but using labeled ester instead, reaches a maximum rate earlier in development (mid-blastula) and thereafter does not decrease through the pluteus stage. Even in the absence of information needed to calculate permeability constants for the ester and for orthophosphate, it appears that the ester penetrates much more slowly. Different mechanisms for the penetration of the two substances probably exist.

The composition of the barium-soluble, alcohol-soluble fraction in this material is unknown. Lindberg (1943) had reported the identification of 1,2-propanediol phosphate in this fraction in sea urchin eggs, but Rudney (1952, 1954) presents cogent reasons for believing this ester separates into the barium-soluble, alcohol-insoluble fraction in rat liver. Sea urchin eggs possess very large amounts of barium-soluble, alcohol-soluble phosphates and of polysaccharides. Interference by the polysaccharides with the clean separation of esters might account for different distribution of propanediol phosphate reported by Lindberg and Rudney. To determine the solubility of propanediol phosphate under the exact conditions used in this investigation, fractionations were made of sea urchin egg homogenates containing known amounts of synthetic 1,2-propanediol phosphate labeled with radioactive phosphorus.

Two separate lots of propanediol phosphate labeled with P^{32} were synthesized by the method described by Lampson and Lardy (1949), using 0.188 and 0.150 millicurie P^{32} in the reaction tubes for the two syntheses. The lead salts obtained were converted to the free acid with dilute sulfuric acid in the first case and H_2S in the other, and remaining traces of lead removed with Dowex-50 in the hydrogen form. The solution of lot 1 was neutralized to pH 7.0. This product contained no inorganic phosphate. The specific activity of the ester was 1439 counts per minute per microgram phosphorus under the standard counting conditions. The second lot, subjected to paper chromatographic analysis, showed a component with the same R_f as a commercial preparation (Nutritional Biochemicals Corp.) and a very faint unidentified second component with much less than 1% of the total activity. No inorganic phosphate was present. The specific activity was not determined.

In each of two separate experiments,[3] a mass of approximately a million unfertilized eggs having an estimated barium-soluble, alcohol-soluble phosphorus content of 300 micrograms was homogenized with cold 0.5 N $HClO_4$. Three hundred to 350 micrograms of phosphorus in the form of labeled propanediol phosphate were added to increase by a significant amount the content of supposed propanediol phosphate already in the eggs. The fractionation was completed in the usual manner and the radioactivity in the various fractions measured with the results given in Table III. Essentially the entire amount of radioactivity was found in the barium-insoluble, plus barium-soluble, alcohol-insoluble fraction. It is concluded, in agree-

[3] We are pleased to acknowledge the help of Miss Kathryn Eschenberg in one of these experiments.

ment with Rudney, that the barium-soluble, alcohol-soluble fraction does not contain propanediol phosphate. This leaves us with no specific information as to the composition of this very large fraction of these eggs.

A preliminary examination of the fraction has been made by paper chromatography. From a number of experiments in which, after isolation, it was subjected to various desalting pre-treatments, evidence for at least three components was derived. In all of these pre-treatments the samples were desalted with Dowex-50 in the hydrogen form and concentrated by evaporation. In some cases the phosphates precipitable by basic lead acetate were chromatographed, and in one the sample was treated with Dowex-2-OH. With different treatments, and depending on the

TABLE III

Recovery of phosphorus-labeled 1,2-propanediol phosphate when added to homogenates of unfertilized eggs in perchloric acid and subjected to barium and alcohol fractionation

Fraction	Experiment 1		Experiment 2	
	cts./min.	% of total activity	cts./min.	% of total activity
Acid-insoluble	31	0.3	38	0.4
Acid-soluble				
Alcohol-insoluble	104	1.0	60	0.7
Barium-insoluble and barium-soluble, alcohol-insoluble	10,203	96.4	8,825	97.8
Barium-soluble, alcohol-soluble	247	2.3	100	1.1
Totals	10,585*	100%	9,023	100%

* 11,174 counts were added to the homogenate. Recovery 94.6%.

amount of sample, one or two clear spots were detectable. Compounds with R_f's of 0.12, 0.29, and 0.71 were found. With this system of chromatography, inorganic phosphate has an R_f of 0.54 and propanediol phosphate, either commercial or prepared by us, has an R_f of 0.73. The similarity between this R_f and that of the unknown at 0.71 is not taken as evidence of identity because other substances, for example glycerol phosphate, have the same R_f in this system.

DISCUSSION

The results of the present investigation indicate that there are at least three components in the barium-soluble, alcohol-soluble fraction. From its magnitude it is possible that one of these could serve as a storage compound of some kind, and the gradual, uniform utilization of the material during development would be in accord with this. The experiments with radioactive orthophosphate demonstrate an appreciable, though not great, synthesis in the fraction, probably in one or more components other than the storage ones.

This synthesis is extremely low prior to fertilization and in the first hour thereafter, but increases demonstrably beginning with the first cleavage. Cleavage initiates an increase in activity in this fraction that is greater than the average increase

for the phosphorus compounds of the egg; before the first cleavage the specific activity in the fraction is 5% of that in the total egg phosphorus, while in subsequent stages it is about 19% of the total. The percentage of radioactive phosphorus that is incorporated into the fraction in embryos of different stages of development after cleavage starts is rather constant, despite large differences in total amount of radiophosphorus that enters the embryo. This suggests that the limiting factor for the synthesis of the component is the availability of phosphate rather than the level of activity of the synthesizing enzymes. Chambers and White (1949) supply evidence that the eggs of this species have a very small inorganic phosphate pool, especially after fertilization, which would be in accord with this idea. The constancy indicates that there are no special periods during development after cleavage in which turnover in this fraction is especially rapid relative to the turnover of the other phosphorus compounds of the embryo.

The evidence used by Lindberg (1946) for identification of propanediol phosphate isolated from cow brain is extensive, but it is not clear in his 1943 or 1946 papers on what basis propanediol phosphate is considered to be a component of the barium-soluble, alcohol-soluble fraction, other than that the fraction has a high stability toward acid and alkaline hydrolysis. His identification of the ester in sea urchin eggs is also based on these features. LePage (1948) identified a phosphate compound in the barium-soluble, alcohol-soluble fraction of rat carcinoma as 1,2-propanediol phosphate on the basis of the phosphorus and lead content of its lead salt and its stability to hydrolysis in 1 N HCl. Against these observations, however, the experiments by Rudney (1952, 1954) and those reported here in which 96% to 98% recovery of labeled, synthetic ester was obtained in the barium-soluble, alcohol-insoluble fraction, demonstrate in a manner that seems unequivocal that this substance is not a component of the barium-soluble, alcohol-soluble fraction. With the demonstration that this ester is not in the barium-soluble, alcohol-soluble fraction, there is, at present, no evidence that propanediol phosphate exists in sea urchin eggs.

The existence of more than one compound in the fraction is further supported by the chromatographic studies which show a minimum of three components. In the related sea urchin, *Strongylocentrotus dröbachiensis,* and in the star fish, *Asterias forbesii,* Mende and Chambers (1953) found that 23% and 60%, respectively, of the phosphorus of this fraction was hydrolyzed to orthophosphate in three hours at 100° C. in 1 N HCl. Further identification or characterization of these components has not been attempted.

In several investigations of the permeability of sea urchin embryos to inorganic phosphate a greatly increased rate of penetration after fertilization has been reported, but subsequent changes in rate have not been followed beyond seven hours in *S. purpuratus* and four or five hours in *Arbacia punctulata.* In these early stages the uptake proceeds at a uniform rate. The experiments reported here show that during later cleavages and blastulation the rate of penetration continues to rise markedly, but that coincident with the onset of the first major form change, gastrulation, the rate shows an abrupt and considerable drop which continues at least to the early pluteus. It will be of interest to determine if this new pattern reflects some profound change at gastrulation either of the metabolism within the cells of the embryo, or of a surface transport mechanism for phosphate correlated with the differentiation of the ectoderm.

SUMMARY

1. The quantity of phosphorus in the barium-soluble, alcohol-soluble fraction of the acid-soluble phosphate compounds of the embryos of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson), was measured until the formation of the early pluteus stage. In two experiments the phosphorus in the fraction decreased by 18.5% and 40.7% in reaching the late prism stage (72 hours).

2. During this period, the total phosphorus of the embryos increased an average of 14.4%.

3. The rate of penetration of radioactive phosphorus from sea water into the embryos during these experiments increased very greatly during cleavage and blastulation, reached a maximum at the onset of gastrulation, and decreased subsequently to a middle level at the prism stage.

4. The rate of uptake of radioactive phosphorus into the barium-soluble, alcohol-soluble fraction was extremely low in unfertilized eggs and in fertilized eggs before the first cleavage, amounting to 0.7% to 1.4% of the total uptake.

5. The rate of uptake of radioactive phosphorus into this fraction in cleaving eggs and embryos mirrored that of the total phosphorus, and the percentage of the labeled phosphorus in the fraction was relatively constant at all stages, averaging 6.5% of the total.

6. Chromatographic examination of the fraction has indicated the existence of at least three components.

7. It is concluded that there are several components in the fraction, at least one of which is a stored material that is steadily metabolized during development, and one or more others synthesized at a rate that is governed largely by the supply of available phosphate.

8. Synthetic radioactive propanediol phosphate added to a perchloric acid homogenate of eggs separated to the extent of 97% into the barium- and alcohol-insoluble, rather than the barium-soluble, alcohol-soluble fraction. It is concluded from this, in contrast to previous reports, that the latter fraction does not contain propanediol phosphate and that no evidence remains for the existence of this ester in sea urchin eggs.

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RESPONSE OF A LIVING ORGANISM, UNDER "CONSTANT CONDITIONS" INCLUDING PRESSURE, TO A BARO-METRIC-PRESSURE-CORRELATED, CYCLIC, EXTERNAL VARIABLE [1]

F. A. BROWN, JR.

Department of Biological Sciences, Northwestern University, Evanston, Illinois

Many overt biological rhythms are known which exhibit cycles of 24 hours with such great precision, at least statistically, that the phases of the cycles do not become significantly altered with respect to external day-night even after weeks or months in conditions of constant darkness and temperature. Such rhythms have been reported in a wide variety of organisms and for many processes (see reviews by Welsh, 1938; Kleitman, 1949; Webb, 1950; Caspers, 1951). Less commonly known is the fact that persistent cycles of lunar-day frequency appear also widespread (*e.g.*, Gompel, 1937; Brown, Fingerman, Sandeen and Webb, 1953; Rao, 1954; and Ralph, 1956). One of the most striking properties of these rhythms appears to be the temperature-independence of their frequency (Brown and Webb, 1948; Brown, Webb, Bennett and Sandeen, 1954; Pittendrigh, 1954). To account for these rhythms, either 1) there must be an astonishingly precise, temperature-independent clock-mechanism present within the organisms, or, 2) they are receiving signals with the same average frequencies from external sources and which serve in some manner as pacemakers, or, 3) some combination of these two. Fluctuations in cycle-length from day to day under constant conditions in such regular overt cycles as running in the mouse (Johnson, 1939), and crab color change (Brown, Webb and Bennett, 1955), despite the great precision of the average lengths of the cycles, suggest strongly the operation of an external pacemaker. Proof for the existence of at least a reasonably precise internal clock has been advanced (Brown, Webb and Bennett, 1955; Renner, 1955) through studying the results of rapid east-west geographic displacement of animals, but it is still not demonstrated that this is sufficiently precise to serve as the exclusive mechanism. Since there are known 24-hour physical cycles which appear to operate on universal time (*e.g.*, atmospheric electrical potential), it may be argued that there is still lacking definitive proof that regular pacemakers are inoperative even during such rapid longitudinal displacement of organisms.

In the course of seeking possible effective external rhythmic signals of the appropriate frequencies, average rhythms of O_2-consumption of primary solar and primary lunar frequencies have been found in all organisms so far examined for them. These have included organisms as widely diverse as seaweed, snails, crabs, Triturus, worms, carrots and potatoes (Brown, Bennett and Webb, 1954; Brown, Webb, Bennett and Sandeen, 1955; Brown, Freeland and Ralph, 1955). Studies of the

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR-122803.

activities of quahogs, oysters and rats indicate that these, too, possess the same general types of average solar and lunar rhythms (Brown, 1954a; Brown, Shriner and Ralph, 1956; Brown, Bennett, Webb and Ralph, 1956).

The fact that there are known to exist average rhythms of barometric pressure of solar- and lunar-day frequencies led to attempts to correlate barometric pressure changes with the observed biological cycles, and some definite correlations were found not only between the hourly rates of barometric pressure change and of concurrent O_2-consumption, or activity, but also between mean daily levels of pressure and mean daily metabolic rates (Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955). There were shown, furthermore, to be such distinct similarities between the general forms of the daily pressure changes and daily variations in oyster and quahog activity (Brown, Bennett, Webb and Ralph, 1956) as to suggest strongly more than a fortuitous relationship. This resemblance was sometimes a direct one and other times one of a mirror image. The experiments to be described herein were initially planned to resolve the problem of a possible direct role of barometric pressure variations.

Materials and Methods

Potatoes, *Solanum tuberosum*, which were purchased at a local grocery store constituted the organism. With a cork-borer, cylinders 22 mm. in diameter and about 1½ cm. high, each bearing an eye, were cut and the injured surfaces allowed to heal. In the experimental situation of constant temperature and constant very low illumination these developed sprouts. They were replaced during the experiments only after long periods when they grew too large for the respirometer vessels.

The respirometers were of the type designed by Brown (1954b) which permitted continuous automatic recording of O_2-consumption. The instrument was modified, however, in such a manner as to permit four respirometers to record as a unit, and the recorder was small enough to be sealed along with the respirometers in a barostat (Fig. 1). The barostat first successfully used was an 11 × 24-inch vertical autoclave sealed with an O-ring. Since it was not possible to view the interior once this was sealed, a simple barograph was included in the system to assure an absence of leakage. Later, five simplified barostats were constructed. These consisted of 1/32-inch copper cylinders, 10¼ inches in diameter and 22 inches deep, with dished bottoms and a 1-inch-wide, ⅛-inch-flat, ground-brass rim. These, covered by twelve-inch vacuum desiccator covers with an electrical inlet for the recorder motor and a glass stopcock passing through a rubber stopper, served as excellent barostats. A ⅜-inch brass tripod ring, fitting snugly within the cylinder served as a platform to support the recorder, with its divers hanging into an enclosed, 10-inch-deep water bath. Each copper cylinder was supported in a 55-gallon steel drum full of water maintained at constant temperature. The temperature settings for the five baths all lay in the range 19.6 to 19.9° C.

The apparatus was shielded from all light except that from a row of 7½-watt, opalescent, incandescent lamps which provided a continuous illumination of about 1–2 ft. c. at the desiccator-cover surface, and obviously some far lower constant illumination at the surface of the organisms.

One cylinder of potato was placed in each of the four respirometers of a recording unit and the whole lowered into a barostat, sealed, and the barostat as-

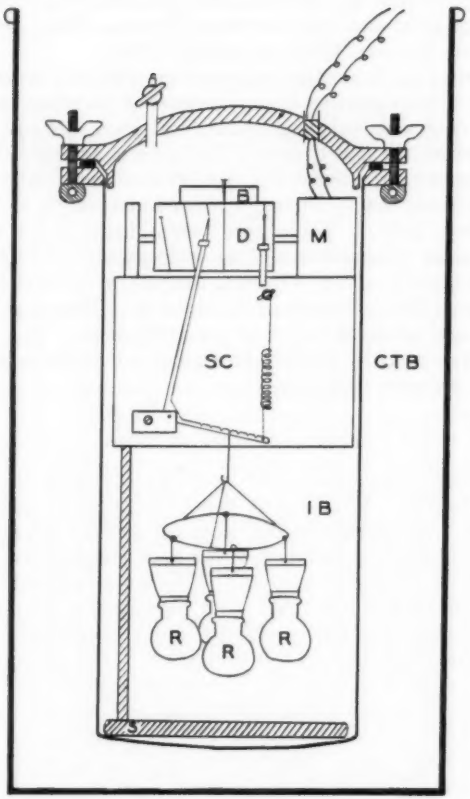

FIGURE 1. Four respirometers (R) hanging into a water bath (IB) from a spring scale (SC) ink recording system registering on a 24-hour drum (D) activated by an electric clock motor (M). A barograph (B) also registers on the same drum. The whole is supported by a stand (S) in a sealed copper-and-brass container which sets deeply in a constant-temperature bath (CTB).

pirated to reduce the internal pressure to 28.00 in Hg. This was sufficiently below the lowest normal external pressure in Evanston, Ill. (ca. 28.5) that the cover would always retain its seal. A barometer in each barostat permitted one to be continuously assured of the absence of leakage. Usually the apparatus was then left undisturbed for three to five days. This was the length of time the recording needle required to spiral down the five-inch-length of the three-inch-diameter brass drum to the recording base-line, with the drum turned one revolution per day with an electric time-clock mechanism. The paper was a smooth-surface, bond, writing paper held in place with rubber cement. The pens were small L-shaped glass capillaries with polished tips and contained a 50–50 glycerine-water mixture, colored with

neutral red. In more recent work, millimeter graph paper and commercially available thermograph pens (counterbalanced) and ink have been found simpler to use.

The springs used were wound from 0.014-inch, 8-18, stainless-steel wire, and were 5 cm. long and about ½ cm. in diameter. Over the recording range (10 gms./cm.) in this mechanical recording system, there was only about a 10% variation from linearity from the first to the last day of recording. There was, undoubtedly, also, a very minor, gradual reduction in pressure in the barostat over a recording period as the O_2-reservoirs of the respirometers were depleted, but this was not significant in view of the large size of the ratio: barostat gaseous volume/O_2-reservoir volume.

Respirometers were always loaded sometime between 8 A.M. and 8 P.M. In the analyses to follow, except when specifically stated otherwise, no data were used before the first midnight after the sealing of a barostat, nor of the day they were opened. Hence, there were usually complete, undisturbed, calendar days of data. All times are central standard.

From April 1 through April 26, 1955, only one barostat was in operation. From April 28 through June 8, five were in continuous operation.

Results

It was apparent even after the first day of recording that the rate of O_2-consumption in the potato is by no means uniform even under constant temperature, illumination, oxygen, CO_2, humidity and pressure. There were fluctuations usually with more than one conspicuous maximum a day. Eight sample days picked at random from the data, illustrating the variation in the two to five separate barostats in which recordings were complete on that particular day, are illustrated in Figure 2. These

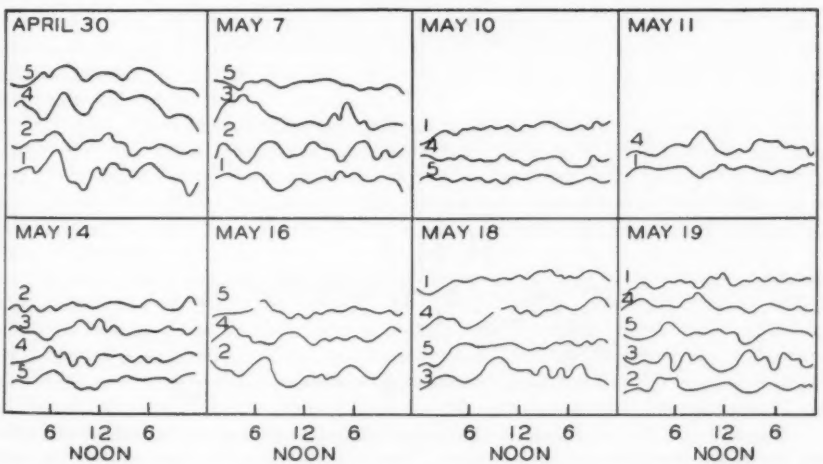

FIGURE 2. The forms of the daily fluctuations in oxygen consumption (three-hour sliding averages) measured on eight randomly selected days in all those (1 to 5) barostats from which records were complete for that day. The ordinate scales are comparable for all, but the patterns have been separated for clarity.

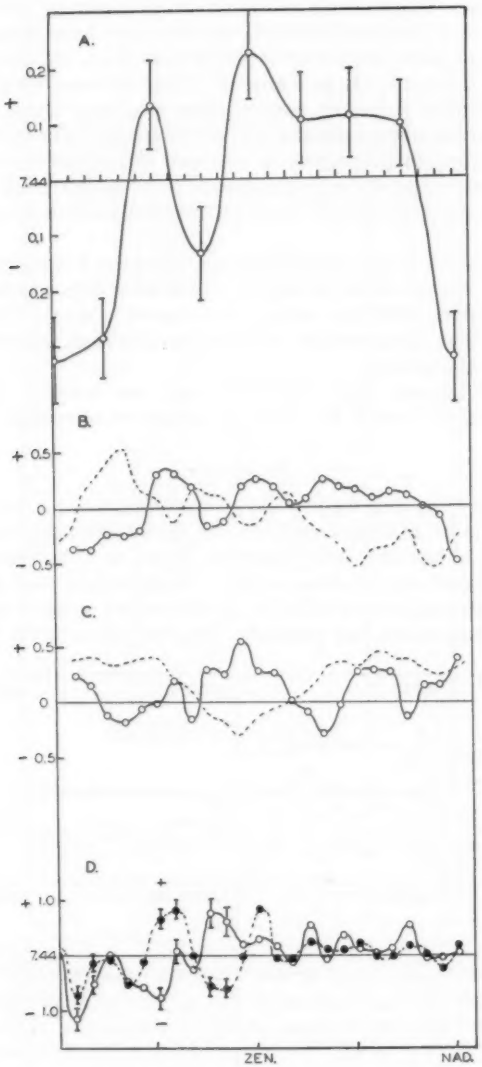

FIGURE 3. A. The mean rates of O_2-consumption for the 8 three-hour periods of the solar-day for all the data of the 154 barostat-days. These are expressed as deviations from the daily means.

B. Moving three-hour means of the daily variation for 100 randomly selected days in 1955 (solid line), compared with the comparable mean daily cycle obtained at the same time of year in 1954.

TABLE I

The percentage increase from lowest to highest values of the daily cycles illustrated in Figure 2

Unit	Date							
	Apr. 30	May 7	May 10	May 11	May 14	May 16	May 18	May 19
1	133	38	71	33			73	49
2	37	73	26		35	84		31
3		67			35		77	52
4	100			46	38	34	89	54
5	67	23	10		80	45	72	32
Av.	82	50	35	40	47	54	78	45

are three-hour moving means. Table I includes the percentage increase from lowest to highest values of the days illustrated. One hundred and fifty-four complete barostat-days of data, including the 29 illustrated in Figure 2, were obtained, together with numerous fractional barostat-days.

Inspection of the daily patterns revealed that there were clearly as many detailed patterns as there were days of data. But there appeared to be a suggestive generic similarity in the records obtained for any given day in entirely independent barostats with respect to amplitude of the fluctuations, gross trends, and approximate times of the major maxima and minima. There seemed, apparently too commonly to be attributed simply to chance, a tendency for one or more of the records to show on any given day an inversion of some of, or even the greater part of, the daily pattern relative to the other concurrent ones. In nearly every instance, however, if one obtained the average daily pattern for a three- to five-day period of continuous recording in one barostat, there was a distinct maximum about 6 or 7 A.M. and a minimum about 9 or 10 A.M.; also, low values nearly always characterized the early morning hours.

In Figure 3A is plotted the mean daily cycle for all barostat-days of data. This is plotted as the mean differences from the mean hourly rate for the whole day for each of the eight three-hour periods of the day. The mean daily fluctuation is seen to be about 8%. The mean hourly value for all these data was 7.44 with the range extending from about 5 to 14. For each value in the figure is indicated the standard error of the mean.

The mean daily pattern is in large measure a mirror image of the one obtained without the use of a barostat during the 29-day period, May 12–June 9, 1954 (Brown, Freeland and Ralph, 1955) involving both inversion of the major 24-hour cycle and the secondary fluctuations superimposed on the larger cycle. Moving three-hour averages of 100 randomly picked days in 1955 and the 1954 cycle are seen in Figure 3B. In 1955 the highest rates for the day occurred in the afternoon; in 1954 the highest rates were found in the morning.

C. The mean lunar day cycle for the potato for the month of May, 1955 (solid line), compared with that obtained in May, 1954 (broken line).

D. The mean daily cycles with sample standard deviations of the means, for the 99 positive barostat-days of O_2-consumption, and the 55 negative ones.

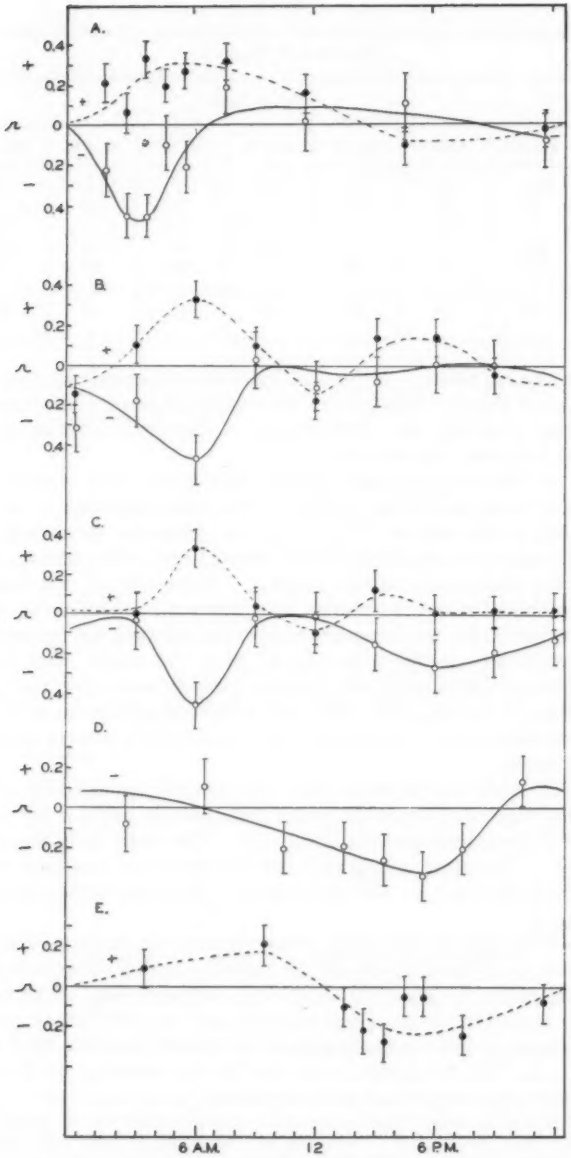

FIGURE 4. A. The correlations between the rate of O_2-consumption during the 4 to 7 A.M. period of a day and the mean rate and direction of barometric pressure change for the same day

There was also an apparent mean lunar-day cycle in the potatoes. It, too, showed a general major inversion relative to the comparable lunar-day cycle obtained in 1954. When only 29 consecutive days of data in May were used, in order exactly to randomize a daily cycle and hence to obtain the minimally distorted form of a lunar-day cycle, the results seen in Figure 3C were obtained. In the same figure is the form of the lunar-day cycle obtained in 1954 without the use of a barostat. These are both three-hour moving means.

Brown, Freeland and Ralph (1955) have shown that for the potato in respirometers subjected to normal fluctuations in pressure in 1954, there was present, even after all appropriate corrections for pressure changes had been made, a correlation coefficient of -0.58 ± 0.035 between the hourly values of O_2-consumption and the concurrent rates and direction of barometric pressure change over a month. That this demonstrated a direct response to some external factor fluctuating with barometric pressure changes was ascertained by obtaining the coefficient for the same monthly period using metabolism on the hours of day n and the pressure changes for day $n + 1$. Now, the value dropped to 0.217 ± 0.038, a value not significantly different from the auto-correlation of hourly pressure changes on two consecutive days. For reasons discussed in that paper it was considered unlikely that the response was a direct one to pressure on the part of the plant. This last presumption is supported amply by this work in which the pressure was kept constant during the recordings.

Although the earlier work had shown a correlation between the hourly rates of O_2-consumption in the potato and the concurrent rate and direction of barometric-pressure change in organisms subjected to barometric-pressure fluctuation, using data available in the current experiments, no correlation with the hourly pressure change in the external environment was found. With 2374 hours of data, a value of r, 0.020 ± 0.021 was obtained.

An attempt was made to test an hypothesis that there was not a correlation with the barometric pressure changes in these data obtained in 1955 because of abrupt 180°-shifts of the phases of at least one important component of daily rhythmicity in the plants. The 154 barostat-days of daily patterns of respiration were, by inspection, divided into two categories on the basis of whether a maximum and minimum occurred about 6 and 9 A.M., respectively, or the cycle at these hours was ap-

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and the two preceding days for various two-hour periods of the day, for both the positive and negative groups of potatoes.

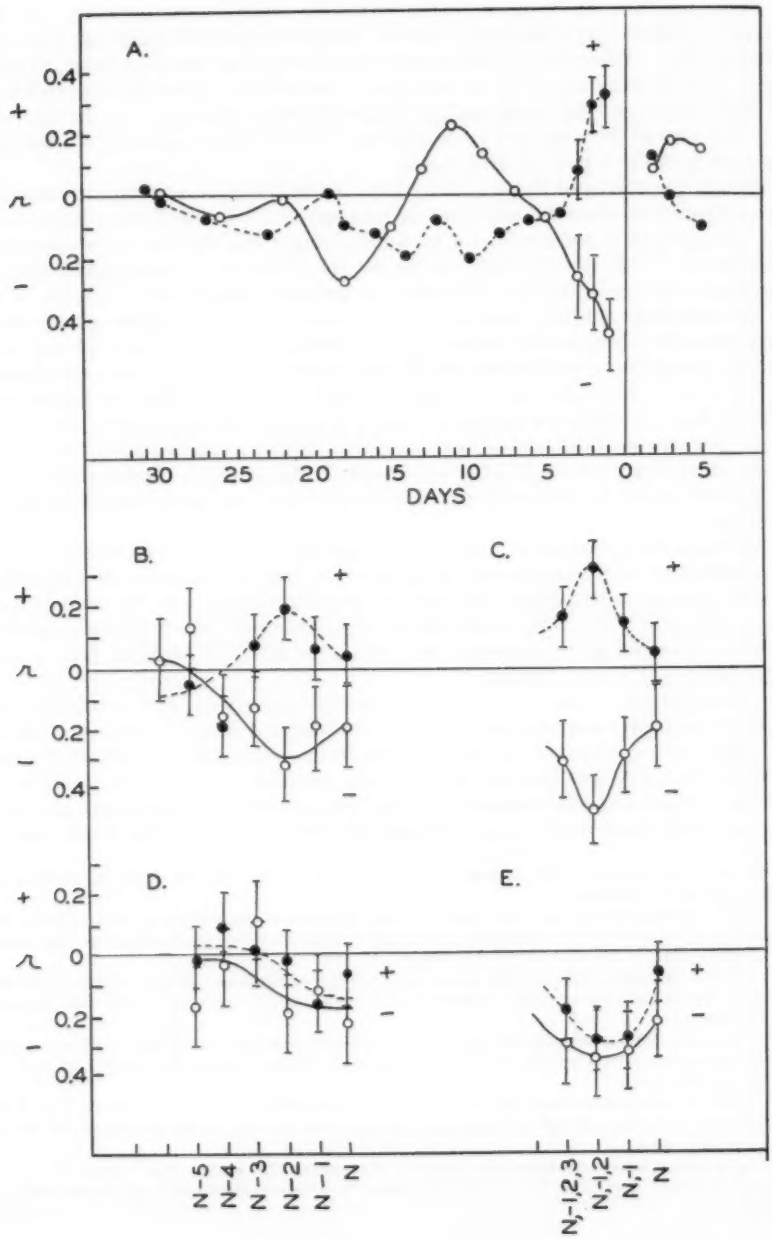
B. The correlations between the mean rate and direction of barometric pressure change during the 2 to 4 A.M. period of a three-day period and the mean rate of O_2-consumption for various three-hour periods on the last of the three days.

C. The correlations between the mean rates of O_2-consumption during various three-hour periods of the day and the mean three-day changes in barometric pressure for the two preceding hours of the day.

D. The correlations between the mean rate of O_2-consumption during the 4 to 7 P.M. period and the mean, three-day changes in barometric pressure during various two-hour times of day, for the negative group.

E. The correlations between the mean rate of O_2-consumption during the period 5 to 8 P.M. and the mean three-day change in barometric pressure for various two-hour periods of the day for the positive group.

The potatoes were maintained in barostats throughout the study. The values of O_2-consumption used are deviations from the daily mean in order to eliminate long-period trends in metabolic rate.


A.
0.4
0.2
0
0.2
0.4
+
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30 25 20 15 10 5 0 5
DAYS
B.
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D.
E.
N-5 N-4 N-3 N-2 N-1 N
N-1,2,3 N-1,2 N-1 N

parently inverted, with a minimum about 6 and a maximum about 9 A.M. The latter were termed the negative group since in the form of the minor fluctuations this was more nearly the form of the 1954 patterns which showed the negative correlation with concurrent barometric pressure changes; the former was termed the positive group.

Barostat No. 1 yielded 14 negative days and 33 positive ones; No. 2 gave 7 negative and 23 positive; No. 3 showed 17 negative and 10 positive; No. 4 had 11 negative and 10 positive; and No. 5 provided 6 negative and 23 positive. Though both positive and negative responses could be found in different barostats on a single calendar-day, there were two or three periods of three or four days, when there seemed to be a high percentage of negative cycles.

In Figure 3D are seen the mean daily patterns for the 99 positive and 55 negative days expressed as mean hourly deviations from the daily means. It is very interesting to note that though these were based exclusively upon selection of an apparent inversion between about 4 and 11 A.M. between the two groups, the remainder of the daily patterns are in extraordinary agreement for the two groups even to most of the minor fluctuations. It was now assumed that, at least for the 4–11 A.M. period, the potatoes were divided into two separate, relatively homogeneous populations with respect to any sign of correlation with any external pressure changes which might be present. On the other hand, it is very important to emphasize here that there was no *a priori* reason why a single value for either of these two groups for any arbitrarily selected single time of day should show any correlation whatsoever with external atmospheric barometric pressure changes.

First, an intensive search was made to learn whether the average rate of O_2-consumption in the 55 days for potatoes of the *negative* group for the 4–7 A.M. period was correlated with external pressure changes in any manner. It was discovered that a correlation, -0.46 ± 0.106, existed between the total respiration at this time on day n, expressed as deviation from the daily mean, and the algebraic sum of the rates of the pressure changes at 2–4 A.M. on days n, $+ (n-1)$, $+ (n-2)$. In Figure 4A it is readily seen that there is a rapid drop to no correlation as one moves away from 2–4 A.M. pressure changes in either direction. In Figure 4A it is also seen that not only did the 99 days of the positive group also show a significant correlation for essentially the same kind of relationship, but with opposite sign, 0.326 ± 0.089. There was similarly a loss of correlation with pressure changes as one moved to other times of day.

FIGURE 5. A. The correlations between the mean deviations in rate of O_2-consumption during the 4 to 7 A.M. period and the mean change in barometric pressure during the 2 to 4 A.M. period during various three-day periods centering on days ranging from 31 days earlier to 5 days later than the day of correlated O_2-consumption, for both positive and negative groups.

B. The correlation between the rate of O_2-consumption during the 4 to 7 A.M. period on day n with the changes in barometric pressure during the 2 to 4 A.M. period on single days ranging from day n to day $n-6$.

C. Correlations between O_2-consumption and barometric pressure for the same hours as in B of the solar day, but now with the means of pressure including increasing numbers of earlier days up to three.

D and E. The same as B and C except that the correlations of pressures at 2 to 4 P.M. with O_2-consumption at 5 to 8 P.M. are used for the positive group and barometric pressure changes between 4 and 6 P.M. are correlated with O_2-consumption for the 4 to 7 P.M. period for the negative group.

Figure 5 demonstrates in two ways that the correlations that have been described are actually the result of a summation by the potato of three or more days of fluctuation in an external factor. First, for both the positive and the negative groups, the correlation not only increases to a maximum as one increases the number of days up to three, but there is a reduction beyond that point (Fig. 5C). Second, it is seen that the best single-day pressure change correlation occurs for both groups with pressure change on day $n - 2$, with lower or no correlation either earlier or later (Fig. 5B). In Figure 5A it is seen that there is a rapid reduction in correlation with summed three-day pressure changes for both positive and negative groups as one attempts the correlations with earlier days or later. The apparent drifts in correlations suggested for three-day periods earlier than $n - 2$, $n - 3$, and $n - 4$, and extending for nearly three weeks probably reflect only auto-correlations of pressures. However, only the correlation with $(n) + (n - 1) + (n - 2)$ is significantly different from 0.

Figure 4B demonstrates that the three-day sums of pressure changes at 2–4 A.M. (days n, $n - 1$, $n - 2$) are not significantly correlated with the rates of respiration (day n) for any time of the day other than the 4–7 A.M. period for either the positive or negative groups. In Figure 4C the rates of O_2-consumption at various periods of day n are correlated with the sums of three-day (day n, $n - 1$ and $n - 2$) pressure changes for the two-hour earlier period. There is a suggestion herein that there may be a second period in the day, in the afternoon, when there is a real correlation for the negative group, though a similar suggestion is lacking for the positive one. When this possibility was explored in detail for the negative group (Fig. 4D), the highest correlation was found between the 4–7 P.M. deviation in O_2-consumption and the 4–6 P.M. (day n, $n - 1$, and $n - 2$) pressure change, -0.347 ± 0.12. There was also found an afternoon correlation for the positive group but not with the same temporal relationship (Fig. 4E). The highest correlation was found between the 2–4 P.M. pressure change and the 5–8 P.M. deviation in respiration, -0.273 ± 0.091. Just as the forms of the mean daily cycles for both groups of potatoes for this part of the days were quite similar, the correlations here also bore the same sign.

It is seen from Figure 5E that the correlation in the afternoon is maximal with the summation of three days (n, $n - 1$, $n - 2$) of pressure changes, with a reduction with fewer or greater number of days. The correlations with single days (Fig. 5D) are relatively small, being about equal for days n, $n - 1$, and $n - 2$.

Evidence supporting the earlier assumption of there being a tendency of the potatoes actually to be reversing from time to time the sign of their response to some external factor became apparent from a study of structure in correlation scatterplots. One of these was the following. When one made no selection whatsoever of the 154 barostat-days of data and determined only the relationship between the extent of the fluctuation, *positive or negative*, from the daily mean for the 5–7 A.M. O_2-consumption on day n, and the algebraic sum of the barometric pressure changes from 2 to 6 A.M. for days n, $n - 1$, and $n - 2$, a coefficient of 0.361 ± 0.070 was obtained. No correlation was obtained unless the sign of the response was ignored.[2]

[2] Further support of this hypothesis of inversions has been obtained since the preparation of this report. With 960 complete potato-days, including hourly data, obtained in barostats during October and November, 1956, the correlation between $3 \times 3 \times 3$-day moving means of the 2 to 6 A.M. barometric pressure change on day n and the three-day moving mean of the 6 A.M. devia-

This highly significant result clearly justified the earlier division of the data into positive and negative cycles. Again, ignoring the sign of deviation of O_2-consumption from daily mean, the correlation between the three-day (n, $n-1$, $n-2$) 5–9 A.M. barometric pressure change and O_2-consumption at 8–11 A.M. yielded a lower value of 0.223 ± 0.077.

There was, further, with no selection of the 154 barostat days of data, found to be a correlation, 0.305 ± 0.073, between the algebraic sum of the 2–6 P.M. barometric changes of day n, $n-1$, and $n-2$ and the deviation in O_2-consumption of the potatoes from the daily mean at the 4–7 P.M. period, if one used simply average rate of pressure change, irrespective of sign. It was evident, however, from examination of a scatterplot that the correlation for those 14 atypical days on which the pressure showed an overall rise at this time of day during the three-day period, was distinctly inferior to that for the 140 days of data of periods for which the pressure was (as typically) falling. The coefficient of correlation between the mean three-day rate of fall in afternoon barometric pressure and 4–7 P.M. deviation in rate of O_2-consumption was 0.355 ± 0.0734.

TABLE II

Correlation between rate of change of barometric pressure and O_2-consumption

Bar. pressure	Potato	Correlation
11–3 A.M.	2–4 A.M.	-0.087 ± 0.08
2–6 A.M.	5–7 A.M.	$\pm 0.361 \pm 0.07$
5–9 A.M.	8–11 A.M.	$\pm 0.223 \pm 0.077$
8–12 A.M.	11–1 noon	$+0.024 \pm 0.08$
11–3 P.M.	2–4 P.M.	-0.056 ± 0.08
2–6 P.M.	4–7 P.M.	-0.305 ± 0.073
5–9 P.M.	8–10 P.M.	-0.084 ± 0.08
8–12 P.M.	11–1 midnight	-0.194 ± 0.078

No other real correlations (except possibly for the 8–12 P.M. pressure change and midnight (11 P.M.–1 A.M.) O_2-consumption) could be found for the potatoes as a whole over the day, even when the structure of correlations was examined for possible reversing responses. The foregoing results are included with some additional ones in Table II.

The potatoes of barostat No. 3 for the 560 hours obtained during May and June showed an hourly correlation of their deviations from the daily means with the concurrent deviations from the daily means of barometric pressure, of $+0.238 \pm 0.040$, a value highly significantly different from zero. That this was a correlation with the concurrent hourly values and not based simply upon similarities of the mean forms of two independent average cycles was readily apparent by finding no correlation for the same period with the pressures of day $n+3$ (0.0923 ± 0.0451), day $n-1$ (0.100 ± 0.0425), and for half the period with day $n+1$ (0.0118 ± 0.059), day

tions from the daily means of $5 \times 3 \times 3$-hour moving means of O_2-consumption on day n, yielded a coefficient of 0.58 ± 0.087. This rather high and unquestionably real correlation indicated that during this 60-day period, the potatoes must have been overwhelmingly of one sign in their correlation with the unknown external factor, namely positive, and, furthermore, this unknown effective force must have retained a high correlation with the morning barometric pressure change during this period. In no other lag or lead relationship, except for smaller and obviously explicable real correlations on days $n+1$ and $n-1$, did there appear from inspection to be correlations significantly different from zero.

$n - 2$ (0.0437 ± 0.059) and day $n - 4$ (0.0622 ± 0.057) of pressure change. The next highest correlation with hourly barometric pressure, and also highly significantly different from zero, was seen for barostat No. 5 for the same period. Barostats No. 1, 2 and 4 showed no comparable correlation. These results, together, clearly suggested that the failures to obtain correlations at some times with barometric pressure were not due to a lack of capacity of the potatoes to respond to a pressure-correlated external factor, but rather due to a failure to observe the response due to a mutual cancelling of opposite signs of response.

Finally, when all the 2976 available hours of data obtained from May 1 through June 8 were correlated with the hourly differences from the daily means for barometric pressure, a value of -0.0697 ± 0.01825 (Fig. 6) was obtained. This value is obviously not zero though very small chiefly because it must be the residual after cancellation of the frequent changes of sign of response to the barometric-pressure-correlated variable. The calculated regressional relationship between the rate of O_2-consumption, and the pressure, for all data, ignoring inversions, shows the rate of O_2-consumption to increase from about 6.8 to about 8.2 arbitrary units, or about

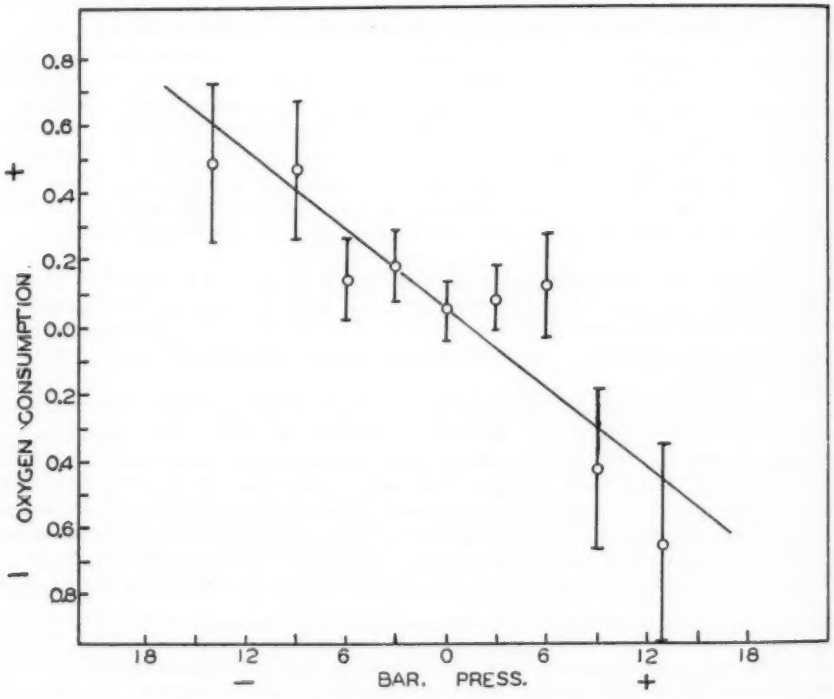

FIGURE 6. The relationship between the deviations from the daily mean of O_2-consumption of five groups of potatoes in barostats between May 1 and June 8, 1955, and the concurrent deviations from the daily mean of barometric pressure ($P < 0.002$).

21%, as the pressure ranges from 0.15 inches Hg above to about 0.15 inch Hg below the daily mean, a moderately large range in the normal pressure changes for a single day.

Discussion

It is quite clear from the preceding account that even when pressure, in addition to light, temperature, humidity and certain other factors are kept constant, there are still substantial fluctuations in O_2-consumption in potatoes. Pressure changes are obviously not the factor responsible. This was strongly suggested in earlier studies (Brown, Freeland and Ralph, 1955) inasmuch as the day-by-day drift in O_2-consumption at 4–7 P.M. appeared to parallel the day-by-day drift in mean barometric pressure, but apparently tending to lead the changes by one or two days.

One might suspect that possibly pressure changes were in some manner responsible for the general inversion of the 1955 cycles relative to the 1954 ones or for the hour-by-hour correlations with pressure change obtained in 1954. That this was not so, has been ascertained through other experiments using the fiddler crab and Fucus in our laboratory during the summer of 1955. These results, to be published elsewhere, show the cycles of these species also to be essentially inverted relative to comparable ones obtained in 1954 and similarly to show no longer the over-all hourly correlation with concurrent pressure changes. With the fiddler crab, however, parallel studies were made under conditions of fluctuating pressure exactly as was done in 1954; the cycles were, like those of the crabs in the barostats, similarly inverted relative to the 1954 ones, and similarly showed no significant over-all hourly correlation with pressure changes. There appears to be only one tenable hypothesis at this time concerning these inversions between the two years, namely, that some significant difference occurred between 1954 and 1955 with whatever fluctuating external factors are responsible for determining the form of the mean biological cycles. All organisms studied in 1955 also appeared to exhibit in some degree, the phenomenon of phase inversions relative to other individuals of the same species being studied concurrently. Whatever the mechanism, it must include to some extent the organism as a biologically responding system. It is possible that various individuals possess different thresholds for some factor which is itself responsible for the changes in sign of response to the external fluctuating factor.

The existence of the inversions which have been described in this report may lead one to question whether these fluctuations in metabolism are strictly rhythmic. Fluctuations in barometric pressure show both solar and lunar tides, but these are, in temperate zones, in good measure obscured by relatively huge climatic fluctuations. The general form of the solar tidal fluctuations may be made evident, however, through the averaging of two to five days of data. This, the potatoes seem also able to accomplish, judging from the results of this study. Hence they are apparently able to exhibit daily and lunar-day cycles of fluctuation, even though these are superimposed upon a much more randomly fluctuating background. But with the more or less erratic sign changes in 1955, the metabolism itself displays a true rhythm only for certain non-inverting components of the solar- and lunar-day cycles. The forms of the cycles are subject to the same fluctuations as are the mean daily pressure cycles. There is always the possibility, however, that the fluctuating factors which produce these responses in organisms, possess some sharper frequency-

determining component than the pressure cycles, from which the organism can obtain an adequate pacemaking signal to regulate their own internal clocks. But just as the large-amplitude random fluctuations in atmospheric pressure make it very difficult to characterize the tides therein, so may externally induced large random fluctuations in the organism tend to obscure a more precise extant organismic metabolic cyclicity.

One should always bear in mind that the metabolic cycles which were the object of this study are not as regularly rhythmic as are numerous overt organismic cycles such as color-change or motor activity in many animals. These latter must be mediated by an internal clock of the same precision as must be postulated to be functioning in these potatoes to integrate the effect of an external stimulus recurring at the same hours of the day over a few days. Such a clock is clearly needed to permit the organism to become an appropriate harmonic analyzer. Such a clock may also act as a buffer to filter out to some extent the large random elements of fluctuation in the external environment. The organism tends to retain for a time and repeat on a 24-hour cyclic basis an environmentally induced pattern of fluctuation.

It is considered highly probable that the responses to the still unknown external factor which are proven to occur by these and earlier experiments in some manner regulate the frequencies of the endogenous clocks.

Summary

1. Fluctuations in O_2-consumption in the potato under constant conditions, including pressure, were observed. The average solar-day cycle was determined and this was found to have in large measure a form which was the mirror image of that obtained during the same months of the preceding year.

2. The average lunar-day cycle was also determined and this, too, was in its principal features an inversion of that found for the same period of the preceding year.

3. A study of the patterns of daily variation for the 154 complete days of data revealed that all the patterns of fluctuation on any given day tended to exhibit a generic similarity to one another, tending to exhibit either parallel, or mirror image fluctuations, and of the same general amplitude.

4. All the daily patterns could be divided in two groups. One group (99 days) called the positive one (since its later discovered correlation with barometric pressure change was positive) possessed a maximum about 6 A.M. and a minimum about 9 A.M.; the negative group (55 days) tended to show the mirror image of this form in the daily period 4 to 11 A.M.

5. The deviation in rate of O_2-consumption from the daily mean for the positive group for the 4–7 A.M. period was found to show a positive correlation with the algebraic sum of the rates of change in barometric pressure during the three preceding 2–4 A.M. periods; the negative group, on the other hand, showed a negative correlation for the comparable relationship.

6. A correlation between the deviation from the daily mean of O_2-consumption at the 4–7 A.M. period and the algebraic sum of the pressure changes for the three preceding 2–4 A.M. periods was found for all 154 barostat-days if one ignored the sign of the deviation in rate of O_2-consumption, proving true an earlier hypothesis

that the sign of the response of the organism to an external pressure-correlated factor changed from time to time.

7. The deviation from the daily mean of O_2-consumption for the 4–7 P.M. period showed a negative correlation with the algebraic sum of the rates of barometric pressure change for the preceding three 2–4 P.M. periods, and the 10–1, midnight deviation, was correlated with the three-day pressure-change from 8–12 P.M.

8. Since it was demonstrated that the correlations were not with single days of an external factor, nor with any averaged three-day periods other than the three immediately preceding daily periods, it was evident that the potatoes were deriving an essential element of the form of their daily fluctuation from a response to an external factor which, since pressure-correlated, clearly possessed average solar-day cycles.

9. The external factor appears to determine in the daily fluctuation of O_2-consumption the amplitude of a morning oscillation (or its mirror image) with about a six-hour period, the height of the late-afternoon maximum, and probably also the extent of the midnight reduction in rate.

10. Possible relationships of the exogenous to endogenous cycles are discussed briefly with reference to the problem of biological clocks.

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THE IMMEDIATE EFFECTS OF LOW DOSES OF X-RADIATION ON THE FREQUENCY OF SEVERAL MITOTIC STAGES IN THE ALLIUM ROOT TIP

ARTHUR C. CHANDLER, JR.[1, 2]

The Department of Zoology and Entomology, The University of Tennessee, Knoxville, Tennessee

Many studies have been made of the effects of radiations on mitosis in plant cells, but the design of the experiments has been such that they could not be compared readily with effects on animal cells. In the present study an attempt has been made to deal with plant material in the same manner as has been frequently used for animal tissues, namely, (1) to maintain the cells at a constant temperature before and after irradiation, (2) to make accurate dosage measurements at each treatment, (3)

TABLE I

The onion root tip cell mitotic cycle at 32° C.

Stage	Criteria by which the beginning of each stage is distinguished in the fixed, stained cell as observed with 4 mm. objective and 10× ocular	Relative frequency
Interphase	Densely granular appearance of nucleus caused by crowding of chromatin threads; nucleoli visible	.7546 (.7552)
Prophase		
Early	Nucleus enlarged; chromatin threads larger and less crowded; granules larger; nucleoli less distinct	.1672 (.1692)
Late	Chromosomes thicker and better separated; nucleoli absent	.0184 (.0182)
Prometaphase	Nuclear membrane absent	.0105 (.0112)
Metaphase	Chromosomes in equatorial plane	.0133 (.0124)
Anaphase	Proximal ends of chromatids separated	.0154 (.0102)
Telophase	Newly-formed cell plate visible; chromosomes less distinct	.0211 (.0211)

Figures in parentheses from Laughlin (1919).

to subdivide certain of the longer mitotic stages, so that more detailed information on the mitotic effect could be obtained, (4) to define carefully the terminology used to designate the different stages (Table I), and (5) to make regular and frequent counts of both control and treated cells.

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[2] Present address: Box 2718, Duke University School of Medicine, Durham, North Carolina.

Materials and Methods

Ease in handling and in control of mold as well as bacterial infection of the roots dictated the use of seeds of *Allium cepa* in preference to bulbs. Preliminary experiments showed no significant differences in mitotic frequency between root tips of different seeds.

Ultraviolet radiation was used to inhibit mold growth during germination. Preliminary tests indicated that the dose of ultraviolet used had no effect on the germination time of the seeds or the rapidity of root growth. Seeds were agitated continuously for a period of six minutes in a quartz flask at a distance of about four inches from a four-watt General Electric germicidal lamp. This was done in a closed, sterilized chamber, which was also used to make subsequent transfers. The seeds were then transferred, aseptically, to sterile petri dishes containing filter paper dampened with distilled water. The petri dishes and filter paper had been sterilized by dry heat and the water had been sterilized by ultraviolet radiation.

A darkened incubator maintained at 26° C. was used to assure constant temperature during germination.[3] After twenty-four hours the seeds were removed from the incubator long enough to receive a second ultraviolet treatment for one minute to further retard the growth of any mold that had succeeded in contaminating the dishes. No roots were in evidence at this time.

After sixty hours, the roots had reached a length of 9 to 15 mm. At this time forty germinated seeds were transferred to each of two petri dishes. One of these sets was used as a control. The other was irradiated with either 128 r or 512 r of x-rays at the same dose rate, *i.e.*, 86 r/min.[4] The 128 r and 512 r doses were chosen because they were not large enough to cause death of the cells but sufficiently large to give easily measurable mitotic inhibition. Irradiation was done with a Coolidge tube (122 kv.p., 5 ma., with a 0.28-mm. aluminum filter). The dose rate was determined with a Victoreen dosimeter before each treatment.

Immediately following x-irradiation, roots of two of the irradiated and two of the control seeds were fixed in a solution of three parts absolute alcohol, one part glacial acetic acid and one part chloroform for six hours. The remaining germinated seeds were immediately placed in an incubator maintained at 32° C. and kept there throughout a five-hour period. During this time control and treated root samples were removed and fixed at one-half-hour intervals.

The material was stained with Feulgen's reagent and made into squash preparations according to a method worked out by Dr. Mary Esther Gaulden of the Oak Ridge National Laboratory (personal communication).

The numbers of the different mitotic stages occurring in ten fields at the center of each root tip squash preparation were recorded. A Howard disc was used to avoid any overlapping of these fields. The number of each of the seven mitotic stages recorded in all tips at a given half-hour interval was summated to give obser-

[3] Preliminary experiments, in which the roots were allowed to grow in darkness at a constant temperature, showed that the diurnal mitotic rhythm, as described by Kellicott (1904), only occurs when *Allium* is allowed to grow in an environment where light is present. These experiments confirm that which was inferred by Gray and Scholes (1951).

[4] This was the dose rate as determined with the dosimeter lying on a wooden table at the same level at which the petri dishes were placed during treatment. The scattering effect of the glass, as determined by measurements made with the dosimeter lying on a petri dish, added approximately 1.3% to these doses and dose rates.

vational totals.[5] All observations were made with a 4 mm. objective and 10 × oculars.

A one-tailed chi-square test was run to determine when the experimental percentages differed, at the 5 per cent level of significance, from the control percentages. Using one degree of freedom, $P > 3.84$ indicates this significance. The results of these tests will be found in Tables II and III.

Results

Results of 128 r x-ray treatment

The effects of 128 r of x-rays on the different mitotic stages are shown in Table II. Interphase was the only stage in which the frequencies of cells showed no significant differences from control frequencies within five hours following x-raying.

Table II

Ratios of experimental frequencies to control frequencies for 128 r

Hours after x-raying	Stage						
	Interphase	Early prophase	Late prophase	Prometa-phase	Metaphase	Anaphase	Telophase
0.0	1.01	0.98	0.95	0.81	0.63	1.12	1.11
0.5	1.02	0.97	1.29	*0.53*	0.77	0.84	0.92
1.0	1.02	0.96	1.16	*0.46*	*0.55*	0.84	0.89
1.5	1.00	*1.15*	1.29	*0.47*	0.60	*0.51*	0.54
2.0	1.02	1.12	0.96	0.65	*0.32*	*0.33*	0.68
2.5	1.04	*1.17*	*0.43*	*0.23*	*0.13*	*0.25*	*0.32*
3.0	0.98	*1.39*	*0.59*	*0.33*	*0.14*	*0.29*	*0.29*
3.5	1.00	*1.44*	*0.20*	*0.05*	*0.07*	*0.14*	*0.13*
4.0	1.00	*1.46*	*0.14*	*0.00*	*0.00*	*0.06*	*0.13*
5.0	1.01	*1.36*	*0.17*	*0.00*	*0.00*	*0.00*	*0.00*

The italicized values indicate that the experimental percentage differs from the control percentage at the five per cent level of significance.

In all other stages, a period of "normal" mitotic activity ranging from a half-hour duration in prometaphase to between two and two and one-half hours in late prophase and telophase was followed by a significant change in frequency. The phrase, "normal mitotic activity," is used to denote the period in which no observable changes in mitotic activity in relation to the control cells was evident.

Early prophase cell frequencies increased significantly beginning one and one-half hours after x-raying, and remained significantly higher than the early prophase control cell counts throughout the five-hour sampling period.

X-rayed late prophases showed no significant differences from the controls for two hours, after which they gradually fell to a minimum at four hours.

[5] These totals may be found in the thesis from which this paper is condensed, in the University of Tennessee library. It is from these data that the ratios in Tables II and III were calculated. Totals at each time interval range from 1296–2420 cells for the controls and 1767–2910 cells for the treated root tips in the 128 r experiment, and from 1340–1742 cells for the controls and 1431–2510 cells for the treated in the 512 r experiment.

Prometaphase, metaphase, anaphase, and telophase treated-to-control ratios decrease more or less gradually, virtually reaching zero three to four hours after treatment.

Results of 512 r x-ray treatment

The effects of 512 r of x-rays on the different mitotic stages are shown in Table III. Following one and one-half hours of normal frequency, treated interphase cells increased in number and maintained a level significantly higher than the controls from two to four hours after treatment.

TABLE III

Ratios of experimental frequencies to control frequencies for 512 r

Hours after x-raying	Stage						
	Interphase	Early prophase	Late prophase	Prometa-phase	Metaphase	Anaphase	Telophase
0.0	1.01	0.96	1.29	1.00	0.89	1.19	1.08
0.5	1.02	0.98	0.91	0.67	1.10	0.95	0.98
1.0	0.99	1.04	1.50	0.86	0.99	0.85	0.97
1.5	1.03	1.03	0.99	*0.31*	*0.43*	*0.50*	0.65
2.0	*1.04*	1.12	0.91	*0.05*	*0.12*	*0.23*	*0.48*
2.5	*1.06*	1.08	*0.53*	*0.05*	*0.00*	*0.09*	*0.26*
3.0	*1.05*	*1.20*	*0.38*	*0.00*	*0.00*	*0.03*	*0.09*
3.5	*1.05*	*1.23*	*0.23*	*0.00*	*0.00*	*0.00*	*0.06*
4.0	*1.06*	*1.21*	*0.16*	*0.00*	*0.00*	*0.02*	*0.00*
5.0	1.02	*1.38*	*0.12*	*0.00*	*0.00*	*0.00*	*0.00*

The italicized values indicate that the experimental percentage differs from the control percentage at the five per cent level of significance.

The early prophase frequency does not differ significantly from the control frequency for two and one-half hours. Subsequently it shows a significantly higher frequency, which is maintained through the five-hour sampling period.

Late prophase, prometaphase, metaphase, anaphase, and telophase frequencies, on the other hand, gradually decrease after one to two hours at the normal level. Late prophases have virtually disappeared at the end of five hours; prometaphases, metaphases, anaphases, and telophases at the end of two to three hours after irradiation.

DISCUSSION

It has been found in a variety of materials, both animal and plant, that if tissues are exposed to ionizing radiations, a decrease in the number of prometaphases, metaphases, anaphases, and telophases follows, and if the dose of ionizing radiations is great enough to reduce the number of cells in these stages to zero, the order of disappearance of these stages will be the same as the order in which cells pass through them (Carlson, 1954). Ionizing radiations tend to cause a blockage in one of the mitotic stages. The cells that have passed this stage continue to progress through the mitotic cycle. Hence, it is obvious that the first stage after the blockage point

will be the first stage vacated by the cells in mitosis, and each subsequent stage would be vacated in the order in which they occur. The more or less gradual progression of cell frequencies toward zero in these stages, as determined in this paper, seems to be in complete agreement with Carlson's statement.

Investigators working with *Vicia* root tips have reported different intervals of time between treatment with ionizing radiations and minimal mitotic activity. Deufel (1951) found that if the broad bean root tip was irradiated with 150 r of x-rays at a dose rate of 5 r/minute, and the tips were kept at 18° C. between treatment and fixation, the low point of mitotic activity was reached after 12 hours. Mottram (1936) states that the minimal mitotic activity of the root tip cells was reached nine hours following a 210 r dose of gamma-rays. According to the work of Juengling and Langendorff (1930), the minimal mitotic rates occur (1) fifteen hours following a 175 r dose of x-rays, (2) eighteen hours following treatment with 420 r of x-rays, and (3) thirty-three hours after irradiation with 550 r of x-rays, but there is no change in mitotic rate after treatment with doses of 40 or 80 r of x-radiation. All of Juengling and Langendorff's work was done with a dose rate of about 20 r/minute.

Working with *Allium* root tips, Darlington and La Cour (1945) wrote that minimal mitotic activity occurs (1) about ten hours after treatment with 150 r of x-rays when the root tips are kept at a temperature of 24° C. after treatment, and (2) about twenty-four hours after x-irradiation with the same dosage if the root tips are kept at 16° C. after treatment. Marshak (1937), however, states that minimal mitotic activity occurs in *Allium* root tips three hours after treatment with either 70 or 220 r of x-rays at a dose rate of 20 r/minute. This is confirmed by Gray *et al.* (1940) using a small dosage of gamma-rays and maintaining a temperature of 25° C. following treatment. Gray, in a personal communication to Carlson (1954), however, states that "the true value could easily have been as late as six hours since the minimum tends to be rather flat."

It has been concluded by Carlson (1942) that the low point of mitotic activity in the grasshopper neuroblast, at 26° C., occurs one hundred minutes after treatment with 31 r of x-radiation. Carlson, Snyder and Hollaender (1949) found that the low point of mitotic activity in the grasshopper neuroblasts is reached about sixty-six minutes after treatment with 32 r of gamma-rays when the material is maintained at 38° C. following treatment. It may be the effect of temperature on the time required for irradiated cells to complete mitosis that accounts for the apparent wide discrepancies in the results cited above.

My studies show that minimal mitotic activity of *Allium* root tip cells occurs between three and four hours after treatment with 128 r of x-rays, and between two and one-half and three hours after treatment with 512 r of x-radiation. The material was maintained at a temperature of 32° C. between treatment and fixation.

Concurrence exists among investigators that, if the treatment dosage is sufficiently large, cells in interphase at the time of treatment may be prevented from entering early prophase. These findings are identical with those in the present study (see Tables II and III).

Differences of opinion, however, exist concerning the immediate reaction of prophase cells to irradiation. Koller (1943), working with *Tradescantia* pollen grains, is among those who believe that prophase is not very sensitive to x-radiation since cells in prophase decrease in frequency following irradiation. These prophase cells,

according to him, complete mitosis with little or no delay as do the cells in prometaphase through telophase. Koller, therefore, concludes that cells in interphase are the most sensitive to ionizing radiations.

By dividing the grasshopper neuroblast prophase stage into five sub-stages, Carlson (1940) concluded from statistical calculations that middle prophase is the stage most sensitive to mitotic inhibition. This same publication also indicated that cells treated while in middle and early prophase revert to earlier stages. This reversion of cells in middle and late prophase was later confirmed by direct observation of living cells (Carlson, 1942). St. Amand (1956) also confirmed this from studies of living cells. Deufel (1951) describes the slowing down of prophases in the *Vicia* root tip after irradiation.

As is shown in Table II, *Allium* root tip cells, treated with 128 r of x-rays, exhibit no significant decrease in late prophase frequency until two and one-half hours after treatment. Obviously, they are not progressing into prometaphase because the frequency of cells in this stage has been decreasing, significantly, since the first one-half hour after treatment, and continue to do so until frequency reaches zero. Therefore, it must be concluded that late prophases have begun to revert, two and one-half hours subsequent to treatment, to early prophase. Early prophase has been increasing, significantly, since one and one-half hours after treatment. This was probably due (1) to an accumulation of cells passing into early prophase from interphase and being, at least partially, blocked from continuing to subsequent stages, and (2) to reversion of late prophases after two hours.

Treatment of the root tip cells with 512 r of x-rays causes this same effect (see Table III) but to a greater degree. Late prophase cells could not be progressing to prometaphase after the third hour, at the latest, as prometaphase frequency is zero after that time. Therefore, as their count is dwindling, late prophase cells must be reverting to early prophase. Some early prophase cells are reverting to interphase as witnessed by a significant experimental increase in interphase cell counts at, and following, the second hour after treatment. The reversion of early prophase cells is indicated by the fact that late prophase is decreasing at the hours when early prophase is showing no significant change. Hence, reversion of early prophase cells would counterbalance the influx of cells into that stage from late prophase. Interphase is increasing significantly at this time. Of course, some of the interphase increase is due to the cells passing from telophase into interphase, but this influx ceases before interphase increase stops.

If these hypotheses and conclusions are justified, they would indicate that late prophase is the most sensitive stage and early prophase is the second most sensitive stage to the effects of x-radiation. Since the criteria I used to designate late prophase seem to include the late and middle prophases of Carlson (1940, 1941, 1942) and St. Amand (1956), the stage sensitivities shown in this paper closely parallel the results reported by these two investigators. Reversion is not a limited phenomenon by any means. Beatty and Beatty (1954a and 1954b) have reported cases of it in *Tradescantia*, while Darlington and La Cour (1945) have noted evidence of reversion in *Trillium*. Reversion of early prophase to interphase may explain the long period in which no change in frequency is observed in early prophase.

The period in which no significant difference between experimental and control frequencies occurred, demonstrated by cells in late prophase, both after 128 r and 512 r doses, could be explained, possibly, in this manner. The critical period must

be in late prophase if reversion occurs as has been previously stated. This period could not be too far into late prophase, however, as no piling-up of cells occurs in this stage. Some cells may pass through this block following treatment to give the "normal" mitotic period in late prophase. This passage will also contribute to the "normal" mitotic periods in subsequent stages for a short time following treatment.

Piling-up in late prophase need not occur, even if the critical period is in late prophase, if all the cells very close to the critical period at the time of irradiation are so sensitive as to revert.

Summary

1. Root tip cells of germinated *Allium* seeds were given doses of 128 or 512 r of x-rays. The mitotic effects of this treatment were determined by making counts, in fixed preparations, of cells in several mitotic stages at regular intervals following x-raying.

2. After treatment with 128 r of x-rays virtual disappearance of cells in late prophase, prometaphase, metaphase, anaphase and telophase was observed between three and four hours. No observable change occurred in the interphase frequencies, but an increase was observed in early prophase one and one-half hours subsequent to treatment.

3. After receiving 512 r of x-radiation, late prophase, prometaphase, metaphase, anaphase and telophase cells fell to minimal frequency between one and one-half hours and three hours. The fall in frequencies after this dose was noticeably more rapid than following the 128 r dose. An increase was noted in interphase frequency two hours following treatment and in early prophase three hours following treatment. Again, late prophase frequency did not reach zero, but was at its lowest point five hours subsequent to treatment.

4. Minimal mitotic activity in the root tip cells of *Allium cepa* is reached between three and four hours subsequent to treatment with 128 r of x-rays, and between two and one-half and three hours following treatment with 512 r of x-rays. Between treatment and fixation all root tips were maintained at 32° C.

5. Reversion of cells from late prophase to early prophase is indicated when the cells are treated with 128 or 512 r of x-radiation and from early prophase to interphase when the cells are treated with 512 r of x-rays. It is probably due to this latter reversion that the minimal mitotic rate was reached in less time by the cells treated with the larger dose of x-rays.

6. Late prophase, which includes the latter portion of middle prophase of certain other investigators, seems to be the most sensitive and early prophase the second most sensitive stage to the mitosis-inhibiting effect of x-radiation.

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LARVAL DEVELOPMENT OF BALANUS EBURNEUS IN THE LABORATORY [1]

JOHN D. COSTLOW, JR. AND C. G. BOOKHOUT

Duke University Marine Laboratory, Beaufort, N. C.; and Department of Zoology, Duke University, Durham, N. C.

Most of the descriptions of larval development of barnacles have been based upon material obtained from the plankton. By this method Willemoes-Suhm (1876) found that *Lepas fascicularis* passed through six free-swimming naupliar stages and one cyprid stage. Since his publication others have made similar studies on different species of acorn barnacles and reported that they may pass through from six to eight naupliar stages and one cyprid stage. Because of the similarity of naupliar and cyprid stages of different species in the plankton the sequence of stages in reconstructions has been questioned. Hence many investigators have attempted to verify reconstructed life cycles by rearing barnacles from the egg to the sessile stage in the laboratory.

Attempts to rear nauplii from unhatched eggs or from naupliar stages in the plankton have met with limited success. Groom (1894a), for example, only maintained *Balanus perforatus* nauplii through the second stage and Treat (1937) was unable to rear *Balanus balanoides* beyond the third stage. Sandison (1954) was also unsuccessful in maintaining several South African barnacles (*Balanus algicola, Balanus amphitrite denticulata, Balanus maxillaris, Balanus trigonus, Chthamalus dentatus, Octomeris angulosa* and *Tetraclita serrata*) beyond the third naupliar stage. Bassindale (1936) was able to rear *Verruca stroemia* to the cyprid stage by feeding the nauplii on *Nitzschia* sp. but the cyprids failed to settle. By using the same methods he could raise *Balanus balanoides* to the fifth naupliar stage only. Batham (1945) maintained the non-feeding nauplii of the goose-barnacle, *Pollicipes spinosus*, from the egg to a stage described as "post-cypris," but no attachment occurred. There have been two definite reports of barnacles being successfully reared from the egg to the settled pin-head: that of Herz (1933) for *Balanus crenatus* and Hudinaga and Kasahara (1941) for *Balanus amphitrite hawaiiensis*. Hudinaga and Kasahara also reared *Tetraclita squamosa* on *Skeletonema costatum* and *Nitzschia closterium* but they all died in the cyprid stage. Pyefinch (1948b, 1949a) refers to culturing and describes the larval stages of *Balanus crenatus*, giving the time of appearance after hatching for the individual stages in the laboratory. He does not discuss the duration of the stages or mention successful attachment and metamorphosis of the cyprid. All investigators to date have reared larvae in mass culture and have determined time and stage of molting by daily sampling. While this method may indicate the number of larval stages it does not give accurately the molting frequency or the variations in intermolt periods within the population nor does it take into account the per cent mortality.

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The barnacle selected for this study was *Balanus eburneus*, for neither has it been reared in the laboratory nor have its larval stages been described, even though it has a range from Massachusetts to South America (Pilsbury, 1916). Its larval history is known only through the report of Grave (1933) who merely stated that it passes through naupliar, metanaupliar, and cyprid stages in 7 to 10 days at Woods Hole, Mass. Therefore, the present study was undertaken to determine the food which would support complete development, the number and description of the larval stages, the frequency of molting, duration and mortality of each intermolt and the length of time required for complete development.

Methods

Intact adult *Balanus eburneus* were removed from pilings and cleaned of attached organisms. In the laboratory the basis of the barnacle was chipped away and the egg lamellae removed. The developing ova obtained and successfully reared had attained the distinct median eye spot and gray color which corresponds to the "H" stage of Groom (1894a). The lamellae were placed in finger bowls containing filtered sea water with a salinity of 28.5 per thousand, *Chlamydomonas* sp., and 200,000 to 400,000 units of penicillin per liter. The bowls were then covered and maintained at 26° C., the average outside water temperature, in a constant-temperature culture cabinet lighted by daylight fluorescent lamps. In order to obtain nauplii of known age only those which were observed to hatch were used. At the time of removal each nauplius was placed in a separate compartment of the rearing assembly. The assembly was made by drilling 100 holes in a piece of ⅜" lucite with a second solid piece of lucite forming the bottom. Each well had a capacity of 1.2 cc. The assembly was then placed in a glass dish, covered, and maintained in the culture cabinet. The contents of the wells were checked two to three times daily with a binocular dissecting microscope. When an exuvium was found it was removed, placed in 70 per cent alcohol, and the time, number of the molt, and mortality recorded for each nauplius. After the second molt freshly fertilized *Arbacia punctulata* eggs were introduced daily into the compartments in addition to the *Chlamydomonas* sp. At this time the larval plutei, developed from the eggs of the previous day, were removed.

The naupliar stages were determined from the number of molts under segregated conditions. These were drawn to scale on graph paper, with the aid of a Whipple disc, from the exuviae, fixed specimens, and, in a few cases, from the living nauplii. The setation formulae were obtained from the exuviae and dissected appendages of known stages. Measurements were made with an ocular micrometer mounted in a compound microscope. In addition to the 121 nauplii raised in individual compartments, hundreds of newly hatched larvae were maintained in finger bowls and plastic compartmented boxes. From these sources specimens were fixed daily in 70 per cent alcohol and 60°-C. Bouin's. After the stages had been determined from exuviae of segregated barnacles the fixed specimens were staged and studied to determine the consistency in appendage setation.

Results and Discussion

The nauplii of *Balanus eburneus*, reared individually, pass through six stages and one cyprid stage, a conclusion which is consistent with Bassindale's (1936) rear-

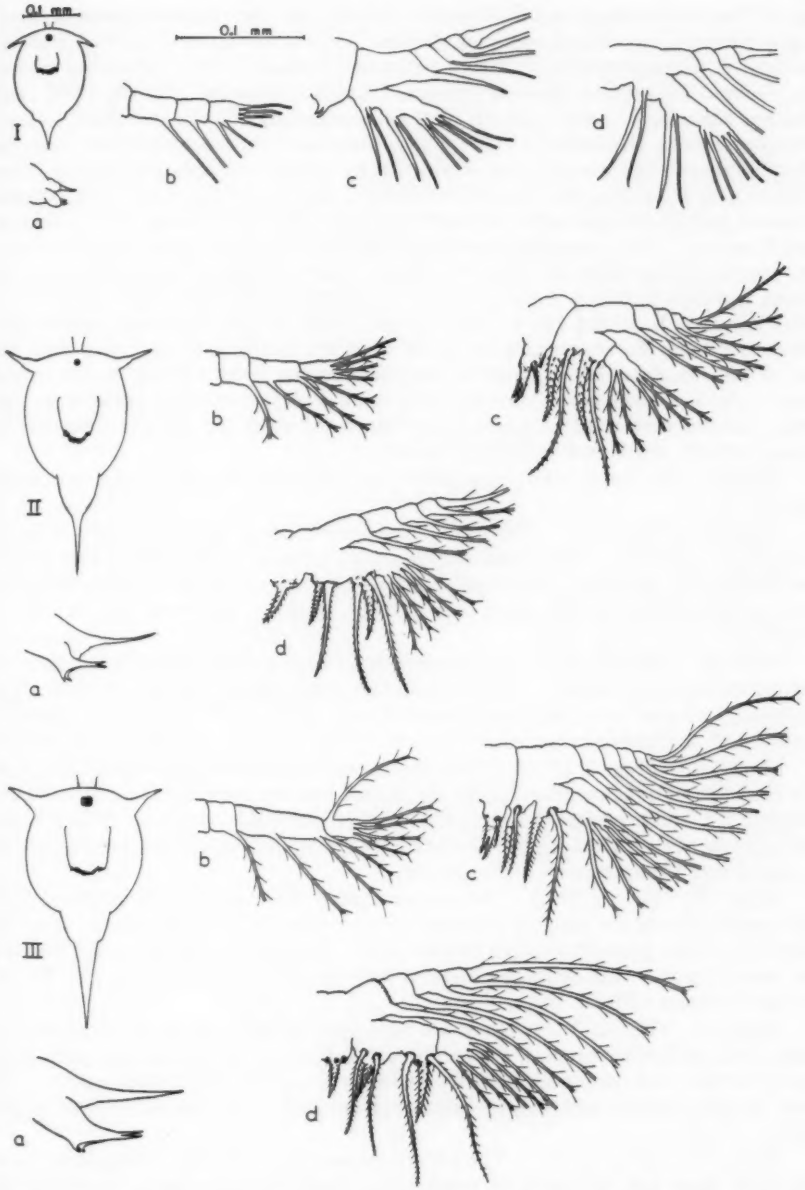

FIGURE 1. Carapace, caudal and abdominal processes, and appendages of naupliar stages I, II, and III of *Balanus eburneus* reared in the laboratory. All swimming setae are cut short. a, lateral view of abdominal and caudal processes; b, antennule; c, antenna; d, mandible.

ing of *Verruca stroemia*, and *Chthamalus stellatus* and the reconstructions of larval stages obtained from the plankton on *Balanus perforatus* (Groom, 1894b), *Balanus balanoides, Balanus crenatus, Verruca stroemia* (Pyefinch, 1948a), *Elminius modestus, Balanus improvisus, Balanus crenatus* (Knight-Jones and Waugh, 1949), and *Balanus algicola, Balanus trigonus, Octomeris angulosa* (Sandison, 1954). However, our results, Bassindale's (1936), and those based on reconstructions from the plankton do not agree with those obtained from culture methods reported by Herz (1933) and Hudinaga and Kasahara (1941). Herz (1933) found that *Balanus crenatus* passed through eight naupliar stages and one cyprid stage and Hudinaga and Kasahara (1941) reported seven naupliar stages and one cyprid stage for *Balanus amphitrite hawaiiensis*. The discrepancy between staged barnacle larvae obtained from the plankton and those taken from mass culture by Herz (1933) and Hudinaga and Kasahara (1941) may be due to the use of appendage setation and other morphological characteristics as the main criteria for staging rather than the use of the exact number of observed naupliar exuviae from the egg to the cyprid stage. As is shown below, setation and spine structure were considered in this study but the number of naupliar stages was based solely on the exact number of molts through which each individual passed.

Nauplii. The most significant characters for each naupliar stage are given below.

Stage I. (Fig. 1, I.) The small frontolateral horns appear slightly recessed and project caudally. The horns are frequently hidden on the ventral side by the antennules and antennae. The caudal process is short and blunt and the abdominal process terminates in two short spines (Fig. 1, Ia). All setae are devoid of setules.

Stage II. (Fig. 1, II.) The frontolateral horns project from the carapace at approximately right angles. The bases of the horns appear slightly swollen. The abdominal process now bears one spine on each side of the base and is approximately half the length of the caudal process (Fig. 1, IIa). All setae bear setules.

Stage III. (Fig. 1, III.) The frontolateral horns are tapered gradually from the junction with the carapace. The abdominal process bears the same spines as in stage II but is now greater than half the length of the caudal process. While larger and slightly heavier than stage II the primary distinguishing features are differences in setation of the antennules and antennae.

Stage IV. (Fig. 2, IV.) The posterior edge of the carapace is delimited from the caudal process for the first time and bears a pair of carapace spines. The abdominal process bears two spines on each side. One pair is located at the base and the second pair in line with the division between the caudal process and the abdominal process (Fig. 2, IVa).

Stage V. (Fig. 2, V.) There are two pairs of spines near the terminal portion of the abdominal process, a large lateral pair and a smaller median pair. Anterior to these is a pair of spines between which is a small, mid-ventral spine. The base is quite swollen and shows partial segmentation. The maxillule first appear (Fig. 2, Va).

Stage VI. (Fig. 2, VI.) The abdominal process is considerably enlarged over the fifth stage and six pairs of small spines mark the developing cirriform appendages beneath the exoskeleton. The spines on each side of the mid-ventral

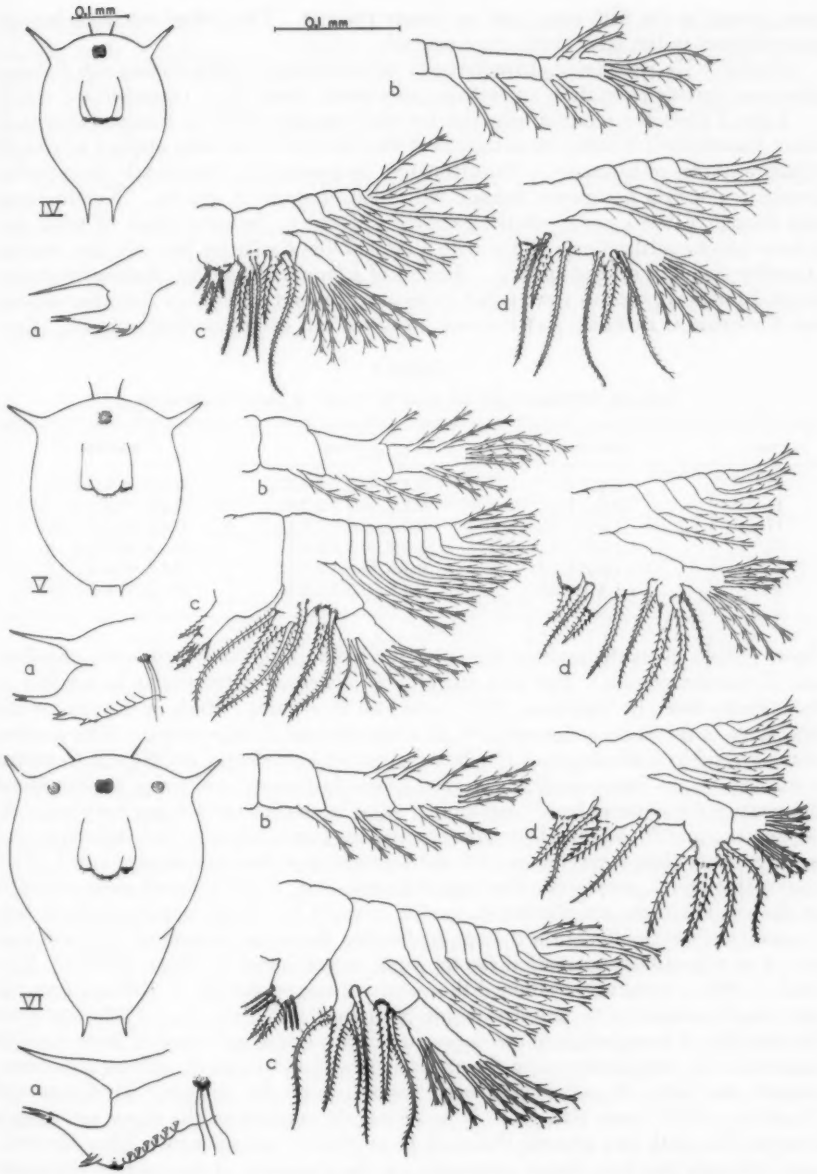

FIGURE 2. Carapace, caudal and abdominal processes, and appendages of naupliar stages IV, V, and VI of *Balanus eburneus* reared in the laboratory. All swimming setae are cut short. a, lateral view of abdominal and caudal processes; b, antennule; c, antenna; d, mandible.

spine, found in the fifth stage, are no longer present. The paired eyespots become quite distinct in the later sixth stage nauplii.

Cyprid. No significant characteristics were observed which distinguish *Balanus eburneus* cyprids from those of *Balanus amphitrite denticulata* (unpublished data).

Table I gives the setation formulae for each naupliar stage of *Balanus eburneus.* Since Bassindale's (1936) introduction of this system it has been applied to nauplii of many species of barnacles. Unfortunately, as asserted by Bassindale, the setation formulae alone do not give a definite indication of stage or species. Knight-Jones and Waugh (1949) point out the extreme differences between types of setae and believe it to be misleading. They note a remarkable similarity between the setation of earlier stages of several species. Stage I of *Elminius modestus, Balanus improvisus* and *Balanus crenatus* were found to be identical and stage II of *Elminius modestus, Chthalamus stellatus,* and *Verruca stroemia* had corresponding setation. San-

TABLE I

Setation formulae of the six naupliar stages of Balanus eburneus

Stage	Antennule	Antenna	Mandible
I	0.4.2.1.1.	0.1.4.–0.3.2.2.2.G.	0.1.3.–0.3.2.2.2.G.
II	0.4.2.1.1.	0.2.4.–0.3.2.2.2.G.	0.1.4.–0.3.2.3.2.G.
III	1.4.2.1.1.	0.2.5.–0.3.2.2.2.G.	0.1.4.–0.3.2.3.2.G.
IV	1.1.4.2.1.1.	0.3.5.–0.5.3.2.4.G.	0.1.4.–0.4.2.4.3.G.
V	1.1.1.4.2.1.1.1.	0.3.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
VI	1.1.1.4.2.1.2.1.	0.4.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.

dison (1954) gives the setation formulae for *Balanus algicola, Octomeris angulosa,* and *Tetraclita serrata.* The first stage of *B. eburneus* corresponds in setation to those forms listed by Sandison (1954) and also *B. crenatus* which in turn makes the first stage of *B. eburneus* identical to *E. modestus* and *B. improvisus.* The setation of the second to sixth stages of *B. eburneus,* to our knowledge, is different from that described for any other species. Norris, Jones, Lovegrove and Crisp (1951) found the setation formulae to be of very limited value in studies on *Balanus perforatus, B. improvisus,* and *B. amphitrite denticulata* and suggest setation to be a developmental feature rather than a specific one for the separation of barnacle larvae. In both *B. improvisus* and *B. amphitrite denticulata* Norris *et al.* (1951) found some variation in the setation of the mandibular exopodite of stage II. They observed that it may "sometimes" resemble the third stage by bearing five setae instead of four and suggest it as a possible explanation for the eight stages found by Herz (1933). Pyefinch (1949a), however, examined thousands of staged nauplii of *Balanus crenatus* and found setation to be uniform within each stage. Norris *et al.* (1951) propose that the rate of morphological development of eyes, limbs, and setae is more strongly influenced by temperature than the rate of growth and onset of ecdysis and consequently the latter do not always occur in step with the former. Hudinaga and Kasahara (1941) note that there is no change in setation of the three appendages between the sixth and seventh stages of *B. amphitrite hawaiiensis.* They discriminate between the two stages primarily on the presence of completely developed paired eyes in the seventh stage as compared to the rudimentary nature in the sixth stage. The present study of *B. eburneus,* in addition to the accounts of other species

by Pyefinch (1949a), Bassindale (1936), and Sandison (1954), shows that the change from rudimentary to completely developed paired eyes takes place in the sixth stage. The development of *B. eburneus*, under controlled temperature conditions, does not reveal any variation in setation within any one stage. Exuviae collected from nauplii undergoing a known molt showed consistency in the setation for that particular stage.

Pyefinch (1949a) describes the third stage of *B. crenatus* as the first one with a delimited carapace bearing a pair of carapace spines. This feature is seen first in the fourth stage of *B. eburneus* and also in *Balanus algicola, Balanus trigonus, Octomeris angulosa* (Sandison, 1954) and *Pollicipes spinosus* (Batham, 1945). The consistency of the mandibular exopodite setation in all 6 naupliar stages of *B. crenatus* appears to be an unusual feature when compared with the increase in setation described for other species.

TABLE II

Measurements of larval stages of Balanus eburneus reared under laboratory conditions

Stage	Carapace		Total length (mm.)
	Width (mm.)	Length (mm.)	
I	0.16–0.18	—	0.19–0.23
II	0.21–0.23	—	0.32–0.34
III	0.23–0.27	—	0.35–0.38
IV	0.27–0.32	0.30–0.33	0.40–0.42
V	0.29–0.34	0.35–0.38	0.44–0.48
VI	0.36–0.39	0.42–0.50	0.54–0.60
Cyprid	—	—	0.46

Table II gives the range in size of the six naupliar stages and one cyprid stage of *B. eburneus*. Knight-Jones and Waugh (1949), using size distribution followed by setation formulae for staging planktonic material, found greater variation in size in the later stages. They also note that "total length" of nauplii is affected by flexing of the abdomen and thus this measurement may be erroneus. Greater variation in size of the later stages was also observed in this study of *B. eburneus* in the laboratory. With setation and observable internal development, such as paired eyes and cirriform appendages, the carapace width and length would be more reliable than total length for staging laboratory reared larvae and presumably planktonic material. The latter study, however, must be made before definite conclusions can be drawn.

Figure 3 gives the variation in duration of intermolt period for the individual stages. Grave (1933) postulated 7 to 10 days as the time required for over-all development of *B. eburneus* at Woods Hole but did not include periods for each stage. Hudinaga and Kasahara (1941) estimated that the individual naupliar stages of *B. amphitrite hawaiiensis* lasted one day each and Pyefinch (1948b), using plankton samples taken at three-day intervals, postulated that each stage of *Balanus balanoides* was three days, or less, in duration. In the present study the duration of the first stage of *B. eburneus* ranged from 15 minutes to 4 hours. Many authors have reported that it is quite short in other species and Hudinaga and Kasahara (1941)

noted that it lasted for 15 minutes to 2 hours for *B. amphitrite hawaiiensis*. The second stage of *B. eburneus* lasts approximately 24 hours and the third 36 hours but with greater variation (Fig. 3). Stage IV averages 2 days, stage V, 2.6 days, and stage VI, 2.5 days but with less uniformity than the earlier stages.

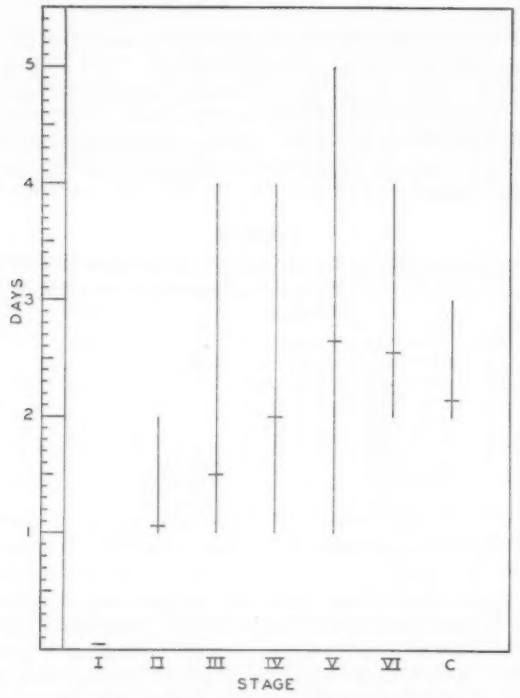

FIGURE 3. Duration of intermolt of naupliar stages (I–VI) and cyprid stage (c) of 121 *Balanus eburneus* reared under segregated conditions. The vertical line indicates the range. The horizontal line indicates the mean.

One hundred per cent of the first stage nauplii underwent ecdyses from 15 minutes to 4 hours after hatching. This was true whether food was available or not. The second stage nauplii molt from 10 to 35 hours after hatching. During this stage the gut appeared green with *Chlamydomonas* sp. The third stage nauplii may molt from the second to fifth days (Fig. 4). As indicated by Sandison (1954) this is a period when many nauplii die if proper conditions are not present. Therefore, it was at this stage that we added to the diet of *Chlamydomonas* sp. fertilized *Arbacia* eggs to furnish food of animal origin. The latter were ingested and the gut took on the echinochrome color of the *Arbacia* eggs. Even so, there was a mortality of 6 per cent (Fig. 5). The fourth naupliar stage shows a greater degree of variation in molting and ecdysis may occur from the third to eighth day with a majority molting on day 4 (Fig. 4). This is accompanied by an increase in mortality (Fig.

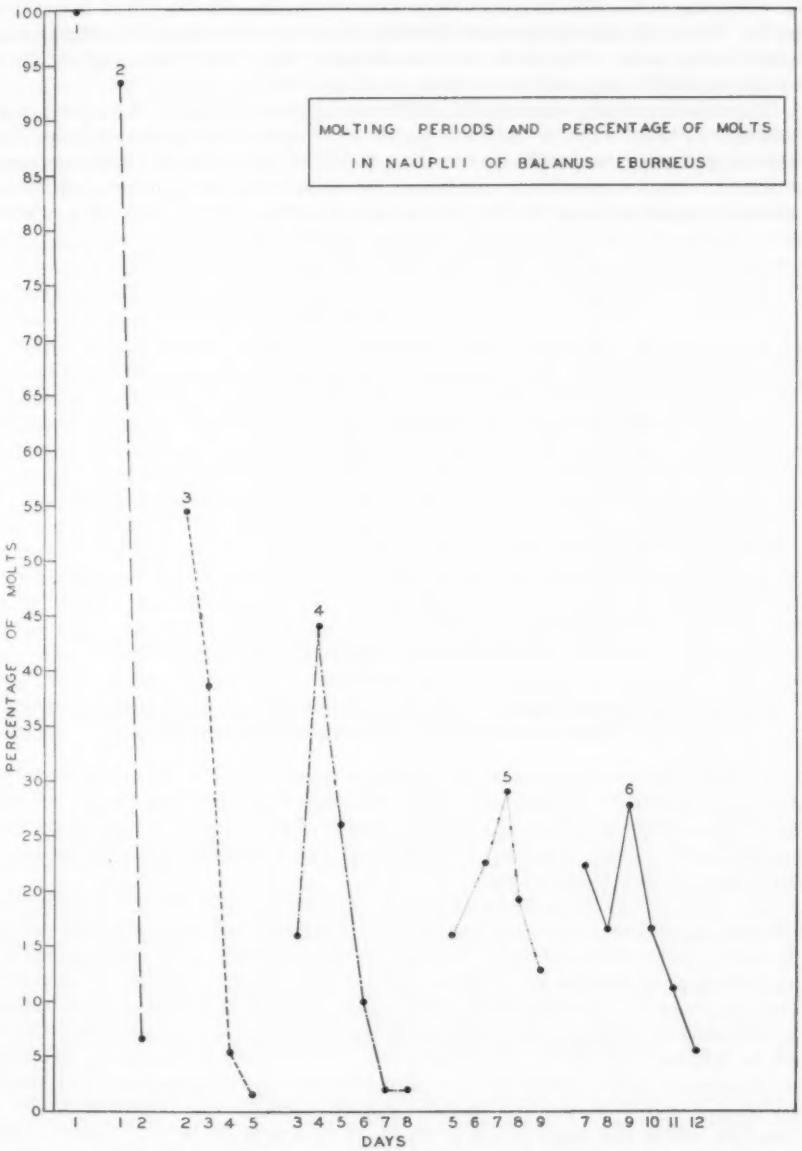

FIGURE 4. Molting frequency of naupliar stages of 121 *Balanus eburneus* reared under segregated conditions. Molts are indicated by numbers.

5). Molting of the fifth naupliar stage occurs from day 5 to 9, with a majority on day 7. This stage showed greater mortality than any other stage, it being approximately 33 per cent. The sixth and final naupliar stage molts into a cyprid on the seventh to twelfth day, with a mortality of 22 per cent.

There is apparently considerable variation in the duration of the cyprid stage. Pyefinch (1948b) reports that the cyprid of *Balanus balanoides* remains free-swimming in laboratory tanks for five days at 4–5° C. but estimated a shorter period in nature. Under laboratory conditions we found that those which settled and underwent metamorphosis to the pinhead did so within one to three days, whereas

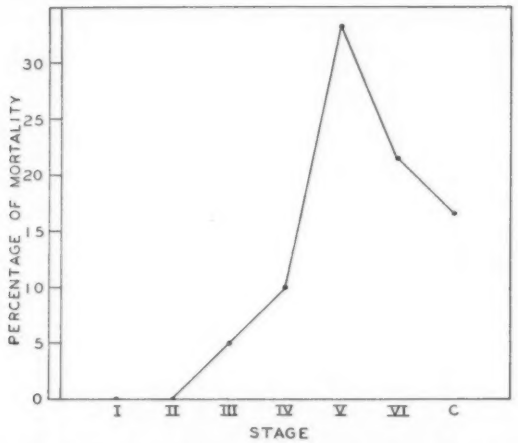

FIGURE 5. Mortality in relation to naupliar stages (I–VI) and cyprid stage (c) of 121 *Balanus eburneus* reared under segregated conditions.

those which persisted as cyprids for longer periods, up to 14 days, failed to settle and died. Mortality in the cyprid stage was approximately 16 per cent. In some cases the mortality was due to incomplete closing of the carapace following the sixth naupliar molt. These abnormal cyprids could live as swimming larvae for two to three days but they failed to settle.

The over-all time of development for *B. eburneus* is quite short when compared with the times given for most other species. Bassindale (1936), although not giving water temperatures, reported 13 days as the minimum time for completion of the sixth stage of *Chthamalus stellatus* and a 22-day minimum for the sixth stage of *Verruca stroemia*. Unfortunately, comparison between species is not too reliable, for the effect of temperature on individual species has not been determined and can only be inferred from studies on other species at different temperatures. Hudinaga and Kasahara (1941) found that the minimum time for development of *B. amphitrite hawaiiensis*, from the first naupliar stage to settling, was 7 days at 23–28° C. This falls within the range found by us for *B. eburneus* at 26° C. Grave (1933) postulated from planktonic material that *B. eburneus* takes 7–10 days for complete development. This figure corresponds with ours even though the temperature at Woods Hole is normally 4 to 8° C. lower than at Beaufort.

The best evidence to date that reduced temperatures increase the time of over-all development of barnacles is shown in the work of Pyefinch (1948b) and Batham (1945). Pyefinch (1948b) found that the over-all development of *B. crenatus* took approximately 30 days at 4–5° C. whereas at 15° C., "or more," the time was reduced to 16 days. Batham (1945) found that the goose-barnacle, *Pollicipes spinosus*, takes 12 days to pass through all larval stages at 18° C. while in the "cold room" (temperature not given) it required 20 to 21 days. The times given by Batham (1945), however, did not include normal completion of the cyprid stage or settling.

The relationships of the substratum and physical factors to settling are outside the scope of this paper. In the present study the only substratum offered was lucite and the physical factors of temperature and light were similar throughout the experiment, in that the temperature was maintained at 26° C. and the rearing assembly received constant illumination from below.

There is always the question of normality when organisms are reared in the laboratory and the query of how survival compares with that in nature. In this experiment if a barnacle completed development, settled, and metamorphosed it was considered normal. Pyefinch (1949b) estimated the survival of barnacle larvae to be between 1 and 9 per cent, depending on the species with which he was working. Bousfield (1955) questions his estimate because of the methods used and was of the opinion that natural survival was approximately 10 per cent. Therefore, our observed survival of 16.3 per cent, under laboratory conditions, is higher than that estimated in nature. Whereas it is undoubtedly true that the chief sources of mortality in nature are dispersal seaward and predation, improper food and bacteria are the chief causes in the laboratory. In preliminary experiments we found that *B. eburneus*, *B. amphitrite denticulata*, and *Chthamalus fragilis* larvae could not be reared beyond the third stage on a diet of *Chlamydomonas* sp. alone. When developing *Arbacia* eggs and penicillin were included, complete development occurred. The actual value of the penicillin is not known but it was observed that the tendency of nauplii to stick to surfaces of the rearing assembly was considerably reduced.

Summary and Conclusions

A technique has been devised for rearing segregated barnacle nauplii, under controlled laboratory conditions, which permits daily observations on the frequency of molting, the number of stages, and the specific characteristics of each stage. From a study of 121 segregated *Balanus eburneus*, plus hundreds in mass culture, reared on *Chlamydomonas* sp. and *Arbacia* larvae at 26° C. the following conclusions may be drawn:

1. Ecdyses provide a definitive method for staging nauplii. The larval phase of *B. eburneus* consists of six naupliar stages and one cyprid stage. Secondary criteria, such as body size, spine structure, and appendage setation, are given for the larval stages.

2. The duration of the six naupliar stages is as follows: first stage, 15 minutes to 4 hours; second stage, one to two days with an average of one day; third stage, one to four days with an average of 1.5 days; fourth stage, one to four days with an average of two days; fifth stage, one to five days with an average of 2.6 days; and the sixth stage, two to four days with an average of 2.5 days.

3. The cyprid stage ranges from one to fourteen days but successful attachment was observed only in those which settled one to three days following the final naupliar molt.

4. The over-all larval development in the laboratory ranges from 7 to 13 days.

5. The first ecdysis occurs from 15 minutes to 4 hours after hatching and is usually followed by the second molt during the first day. The third molt occurs from the second to the fifth days and the fourth molt takes place from the third to the eighth day. The fifth ecdysis occurs from the fifth to the ninth day with the sixth molt ranging from the seventh to the twelfth day.

6. Tables are given for the size of the nauplii and the setation of the appendages.

7. Successful metamorphosis and attachment was observed in 16.3 per cent of the 121 barnacle nauplii studied under segregated laboratory conditions.

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AMINO ACID CONTENT OF MARINE BORERS

RICHARD W. DRISKO AND HARRY HOCHMAN

Chemistry Division, U. S. Naval Civil Engineering Research and Evaluation Laboratory, Port Hueneme, California

Although a great amount of study on the prevention of marine borer attack on wooden structures has been made in the last century, relatively little is known of the biochemical make-up of these organisms. The only published investigation of the amino acid content seems to be that of Lasker and Lane (1953) who reported eight amino acids present in *Teredo bartschi* Clapp. In view of the current idea (Fox, 1956) that primitive organisms have proteinaceous matter similar to that found in higher forms of life, it does not seem likely that several commonly occurring amino acids would be absent from the protein structure of *Teredo*.

In the present investigation *Teredo diegensis, Bankia setacea* (another member of the Teredinidae family) and two species of the crustacean borer *Limnoria* were investigated to determine their amino acid content. In all cases considerably more than the eight amino acids reported by Lasker and Lane were found to be present. In addition, southern yellow pine, redwood, and greenheart (*Ocotea rodiaei*) were hydrolyzed for identification of the amino acids present. The latter species of wood has been reported to possess a high protein content (Marchán, 1946) and to be highly resistant to marine borer attack (Baldwin, 1938).

Materials and Methods

The marine borers investigated were *Teredo diegensis, Bankia setacea,* and *Limnoria tripunctata* and *quadripunctata*. The first two were removed from both pine and redwood blocks, the *Limnoria tripunctata* from a creosoted log, and the *quadripunctata* from a redwood block in the same waters. Whole adult animals taken from the harbor and *Teredo* larvae produced in the laboratory were hydrolyzed. The larvae were collected by screening the overflow sea water from a tank containing pine blocks infested with *Teredo*. In addition to analyzing whole animals, *Bankia* was dissected into viscera, mantle, and gill, and *Teredo* into viscera, mantle, and reproductive organ containing larvae, and each section was analyzed individually. These dissected parts were extracted with 80% alcohol prior to hydrolysis in order to separate the free amino acids from those bound in the protein structure of the animals. The extracts were concentrated, the fatty substances extracted with chloroform, and the residues spotted for chromatography without further treatment.

Acid hydrolysis was in all cases effected by heating with 6 N HCl in a boiling water bath for twenty hours. Excess of HCl was removed by repeated evaporation to dryness under reduced pressure. Two-dimensional chromatograms using Whatman No. 1 filter paper with phenol solvent (Block, 1950) in the first direction and lutidine-collidine (Dent *et al.*, 1947) in the second were routinely run by the capil-

lary ascent method of Williams and Kirby (1948). The leucines were separated on a one-dimensional descending chromatogram using water saturated *tert*-amyl alcohol in an atmosphere of diethylamine. Confirmation of the identity of each spot was made by running suitable quantities of authentic amino acids simultaneously. The amino acids were revealed with the ninhydrin reagent of Levy and Chung (1953). Histidine and tyrosine were further identified by Block's (Block *et al.*, 1952) modification of the Pauly reaction; arginine, by one-dimensional chromatography using the solvent system of 77% alcohol and diethylamine (Block and Bolling, 1951); and the sulfur-containing amino acids, with the platinic iodide reagent (Toennies and Kolb, 1951). Dent's (1948) hydrogen peroxide treatment likewise proved to be useful in identifying the sulfur-containing amino acids.

A spot corresponding to lanthionine was eluted by the method of Dent (1947) from an unsprayed two-dimensional chromatogram. It was then spotted on a one-dimensional descending paper and run for five days using *n*-butyl alcohol-acetic acid (Partridge, 1948) as the solvent.

Glutamine, which was found to occur as a free amino acid in the Teredinidae, was further identified by elution (Dent, 1947) from an unsprayed two-dimensional chromatogram, by hydrolysis with acid, and by rechromatography. Glutamic acid was the only amino acid found on this latter chromatogram.

The barium hydroxide hydrolysis method of Levy and Chung (1953) was used to detect tryptophan which is unstable to acid hydrolysis.

The three woods investigated were hydrolyzed with 6 N HCl in a boiling water bath for twenty hours. Before spotting, each hydrolyzate was partially purified by adsorption on a column of the hydrogen form of Dowex 50-X8 (200 to 400 mesh), washing with water, and elution with 4 N ammonia. A synthetic mixture of amino acids was similarly treated.

Results

The amino acids found in the acid hydrolyzates of whole living organisms are summarized in Table I for each of the species studied. Taurine is also listed in this Table, as its spot was readily detected in these hydrolyzates. When the freshly-ground tissues of the Teredinidae were treated with 80% alcohol, taurine and β-alanine were extracted into the alcohol. All other amino acids listed in Table I were found in the hydrolyzates of the 80% alcohol-insoluble residues.

The alcoholic extracts of the dissected Teredinidae fragments all gave readily identifiable spots for α-alanine, β-alanine, glutamic acid, glycine, and taurine. In addition, some of the fragments, notably the mantle, gave very faint tests for some of the other amino acids in Table I, but these were not further investigated. The *Bankia* gill, the *Teredo* reproductive organ, and the mantles from both organisms were found to contain glutamine.

The acid hydrolyzates of both species of Teredinidae removed from redwood gave a spot corresponding to lanthionine, while those from pine did not. The spot gave a positive sulfur test with the platinic iodide reagent and proved to be chromatographically indistinguishable from lanthionine in the solvent systems used.

An unidentified spot was found in the acid hydrolyzates of both species of *Limnoria*. It is designated "A" in Table I. The spot persisted even when the tissues were hydrolyzed for an additional twenty-four hours. It was located on a

TABLE I

Amino acids in acid hydrolyzates of whole organisms

Amino acid	TP	TR	TL	BP	BR	LT	LQ
α-Alanine*	X	X	X	X	X	X	X
β-Alanine	X	X	X	X	X	O	O
Arginine*	X	X	X	X	X	X	X
Aspartic acid	X	X	X	X	X	X	X
Cystine-cysteine	X	X	X	X	X	X	X
Glutamic acid	X	X	X	X	X	X	X
Glycine	X	X	X	X	X	X	X
Histidine	X	X	X	X	X	X	X
Isoleucine	X	X	X	X	X	X	X
"Lanthionine"	O	X	O	O	X	O	O
Leucine*	X	X	X	X	X	X	X
Lysine	X	X	X	X	X	X	X
Methionine*	X	X	X	X	X	X	X
Phenylalanine*	X	X	X	X	X	X	X
Proline*	X	X	X	X	X	X	X
Serine	X	X	X	X	X	X	X
Taurine	X	X	X	X	X	X	X
Threonine	X	X	X	X	X	X	X
Tyrosine*	X	X	X	X	X	X	X
Valine*	X	X	X	X	X	X	X
Spot "A"	O	O	O	O	O	X	X

* Reported by Lasker and Lane to be present in *Teredo bartschi* Clapp.

X Present in readily detectable amounts.

O Not present in readily detectable amounts.

TP—*Teredo* from pine; TR—*Teredo* from redwood; TL—*Teredo* larvae; BP—*Bankia* from pine; BR—*Bankia* from redwood; LT—*Limnoria tripunctata*; LQ—*Limnoria quadripunctata*.

two-dimensional phenol and collidine paper between the spots for threonine and tyrosine. The R_f value in phenol was considerably lower when run in an atmosphere of acetic acid (Dent, 1948) than in an atmosphere of ammonia.

Tryptophan was not found in any of the alkaline hydrolyzates. This may have been because of its relative insensitivity to the ninhydrin reagent as compared to other commonly occurring amino acids.

The amino acids found in the three species of wood are listed in Table II. The greenheart hydrolyzate gave a much stronger phenylalanine spot than did the hydrolyzates of either pine or redwood. When a synthetic mixture of amino acids was treated with Dowex-50, all but lanthionine were eluted with ammonia.

DISCUSSION

These data indicate that there are at least eleven amino acids present in *Teredo diegensis* in addition to the eight reported present in *Teredo bartschi* Clapp by Lasker and Lane. It is considered unlikely that so many additional amino acids would occur in the one species and not the other.

The fact that taurine and β-alanine are not present in any of the hydrolyzates of tissues that had been extracted with 80% alcohol indicates that these compounds are

not present in the protein of the borers investigated. Seventeen amino acids are common to each species. All the amino acids seem to be distributed throughout all the Teredinidae sections. In addition, spot "A" was detected in the hydrolyzates of both species of *Limnoria*, and a spot corresponding to lanthionine was found in the hydrolyzates of both species of Teredinidae which had been removed from redwood. A corresponding spot was not found in the hydrolyzates of animals removed from pine. Cystathionine is reported (Dent, 1948) to have chromatographic properties similar to those of lanthionine. Neither of these two amino acids has been found in nature (Dent, 1948).

TABLE II

Amino acids readily detected in acid hydrolyzates of woods studied

α-Alanine	Lysine
Aspartic acid	Phenylalanine
Glutamic acid	Proline
Glycine	Serine
Hydroxyproline	Threonine
Isoleucine	Valine
Leucine	

The three species of wood studied contain thirteen amino acids in common. Hydroxyproline was found in all three woods but in none of the marine borers. A spot corresponding to lanthionine was not found in the chromatograms of the acid hydrolyzed redwood, but it was shown that lanthionine is not eluted with the other amino acids when a synthetic mixture is similarly treated.

SUMMARY

1. Eighteen naturally-occurring amino acids and taurine have been identified chromatographically in the acid hydrolyzate of *Teredo diegensis* and *Bankia setacea*, and seventeen naturally-occurring amino acids and taurine have been identified chromatographically in *Limnoria tripunctata* and *quadripunctata*. A spot corresponding to lanthionine has been found in the above Teredinidae living in redwood blocks but not in those living in pine blocks. Glutamine has been found in the alcoholic extract of freshly-ground Teredinidae sections.

2. Three species of wood have been hydrolyzed with acid, and all were found to contain the same thirteen amino acids.

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THE RATE OF FEEDING OF THE COMMON OYSTER DRILL, UROSALPINX CINEREA (SAY), AT CONTROLLED WATER TEMPERATURES

JAMES E. HANKS[1]

U. S. Fish and Wildlife Service, Milford, Conn.

Information on the voracity of the oyster drill, *Urosalpinx cinerea* (Say), within certain ranges of water temperature has been reported by a number of investigators, but evaluation of their destructiveness at specific temperatures maintained within close limits is virtually non-existent. In addition, the conclusions regarding the destructiveness of drills within the different temperature ranges are drawn from observations made at widely separated geographical areas (Carriker, 1955).

Observations by Stauber (1950) and Loosanoff and Davis (1950–51) on certain activities of drills from different areas of the North Atlantic Coast suggest the existence of distinct physiological races. If such races exist, a temperature-dependent activity, such as feeding, determined for drills from one area would not necessarily be applicable to drills of another habitat. Therefore, we have attempted to establish the feeding rate of a single geographical population of oyster drills at several constant temperatures. It was felt that such a study would not only provide more detailed information about the predation of drills, but also present a basis of comparison for investigators interested in the problem of physiological races.

Methods

A bank of wooden frames, constructed to hold a number of enamel trays (18″ × 20″ × 3″), was arranged on a laboratory water table. Each tray was supplied with a separate, continuous flow of sea water. Temperatures were adjusted by mixing cold and heated sea water in glass cylinders, just above the frames, giving each bank of trays a separate supply of water at a constant temperature (Loosanoff, 1949). Water in the trays was maintained at approximately ± 1.0° C. of the temperature desired.

Since the drills had a tendency to move up the sides of the tray and leave the water, covers were made to fit into each tray to keep the drills below the water line. They were constructed of Lucite plastic frames with a covering of ⅛″-mesh Saran plastic screening.

The salinity of the water deviated only slightly from 25‰ throughout the experiments and is, therefore, considered to have exerted no influence on differential feeding at the various temperatures.

To obtain a more comprehensive evaluation of feeding by drills at different temperatures, two species of bivalves, the common oyster, *Crassostrea virginica* Gmelin, and the mussel, *Mytilus edulis* Linné, were used as foods in separate experiments.

[1] Present address: Department of Zoology, University of New Hampshire, Durham, New Hampshire.

Each tray contained 20 adult, Long Island Sound drills measuring from 20.0 to 25.0 mm. in height, and either 30 to 40 oyster spat, ranging in size from 10.0 to 30.0 mm., or 40 mussels, ranging in size from 20.0 to 30.0 mm. Spat were growing on cultch (old oyster shells) in such numbers that four to five shells supplied the total needed. The oyster spat clusters or mussels were so distributed about the tray that no large concentration of food occurred at any one point. The drills were dispersed throughout the tray to decrease the opportunity for feeding by two or more drills on the same bivalve. At no time during the experiments did the drills consume all of the bivalves available in any tray, thereby indicating that feeding was maximal for each feeding period.

The drills were brought to the experimental temperatures by keeping them for two to three hours at each five-degree level until the desired temperature was reached. After the experiment was begun the number of bored bivalves, as well as the number and condition of drills present, was ascertained at regular intervals. Following each such examination, oyster spat or mussels were added to replace those destroyed by the drills. Mortality of drills was low at all temperatures, except 30.0° C. All dead drills were replaced by drills from stocks kept at the same water temperature as the experimental groups to assure that their prior conditioning was the same.

Results

The feeding rates are expressed as the number of bivalves destroyed per drill during one week of feeding. Since low temperatures could not be maintained during the spring and summer, observations on the groups at 5.0° C. were discontinued after 52 days, on the 10.0° C. groups, at 69 days, and on the 15.0° C. groups, at 90 days; while the groups at 20.0°, 25.0° and 30.0° C. were under observation for 102 days (Table I).

At 5.0° C. the drills did not feed, nor did they show any tendency to attack the spat during the 52 days of the experiment. They usually remained in groups, each

Table I

Feeding rate of U. cinerea on oyster spat, size range 10–30 mm., at controlled water temperatures

Temperature ° C.....	5.0		10.0		15.0		20.0		25.0		30.0	
Tray.....	1	2	1	2	1	2	1	2	1	2	1	2
No. of drills	20	20	20	20	20	20	20	20	20	20	20	20
Feeding period (days)	52	52	69	69	90	90	102	102	102	102	102	102
No. of spat consumed	0	0	49	41	124	133	313	355	429	398	285	327
Spat consumed per drill per week	0	0	0.25	0.21	0.48	0.52	1.07	1.21	1.47	1.37	0.98	1.12

drill with the foot extended and attached firmly to the tray. Movement of a few inches by some drills, as shown by mucus paths, was noted on several occasions.

Feeding at 10.0° C. appeared to be limited to occasional attacks by individual drills since the rate of feeding was only about one spat per drill every four to five weeks. The rate of feeding on oyster spat increased as the temperature increased from 10.0° to 25.0° C. The rate approximately doubled for each five-degree increase in water temperature from 10.0° to 20.0° C. However, the next five-degree rise, to 25.0° C., increased the rate of feeding only about 25 per cent over that at 20.0° C., and a distinct decrease in the rate of feeding occurred at 30.0° C. Thus, the optimum temperature for feeding of drills on oyster spat was at or near 25.0° C.

The drills maintained at 30.0° C. were also slower in turning over and attaching to the tray than those kept at 25.0° C. On two occasions, when the temperature rose to about 34.0° C., 70 to 80 per cent of the drills were killed, although in neither

TABLE II

Feeding rate of U. cinerea on mussels, size range 20–30 mm., at controlled water temperatures

Temperature ° C.	10.0		15.0		20.0		25.0		30.0	
Tray	1	2	1	2	1	2	1	2	1	2
No. of drills	20	20	20	20	20	20	20	20	20	20
Feeding period (days)	34	34	57	57	57	57	44	44	44	44
No. of mussels consumed	1	4	39	32	88	73	101	109	89	105
Mussels consumed per drill per week	0.01	0.04	0.24	0.20	0.54	0.45	0.80	0.87	0.71	0.82

instance were they exposed to this temperature for more than 16 hours. The surviving drills were not attached to the tray or to the oyster spat, as they usually were at 30.0° C. These observations suggest that while drills do feed at 30.0° C., this approaches the lethal temperature level.

Comparable data for oyster drills feeding on mussels show that the optimum feeding temperature again lies near 25.0° C. (Table II). Apparently, only a few of all the drills kept at 10.0° C. fed during the 34 days of the experiment. The rate of feeding at 15.0° C. approximated one mussel per drill every five weeks. This rate was about doubled for each five-degree rise in temperature from 15.0° to 25.0° C. Although the rate of feeding at 30.0° C. was lower than that at 25.0° C., it was not as low as would be expected from the results when oyster spat were used as food. Probably, the rate of drill feeding at 30.0° C., in the mussel-fed experiment, was somewhat higher due to differences in handling. It was necessary to examine each tray and change the mussels daily to avoid a high mortality and resultant bacterial decomposition of mussels at 30.0° C. Thus, the drills may have been induced to attack more mussels because they were unable to feed without interruption.

Statistical treatment of the data by analysis of variance indicates that differences in feeding rate between temperature levels are highly significant ($F = 85.96$ with $df = 4,5$ for mussels as food and $F = 143.84$ with $df = 5,6$ for oyster spat as food; both values being significant beyond the 0.001 level). In order to determine which of the differences between temperatures were significant, a test for significance of gaps between means was applied (Bliss and Calhoun, 1954). Results of this test

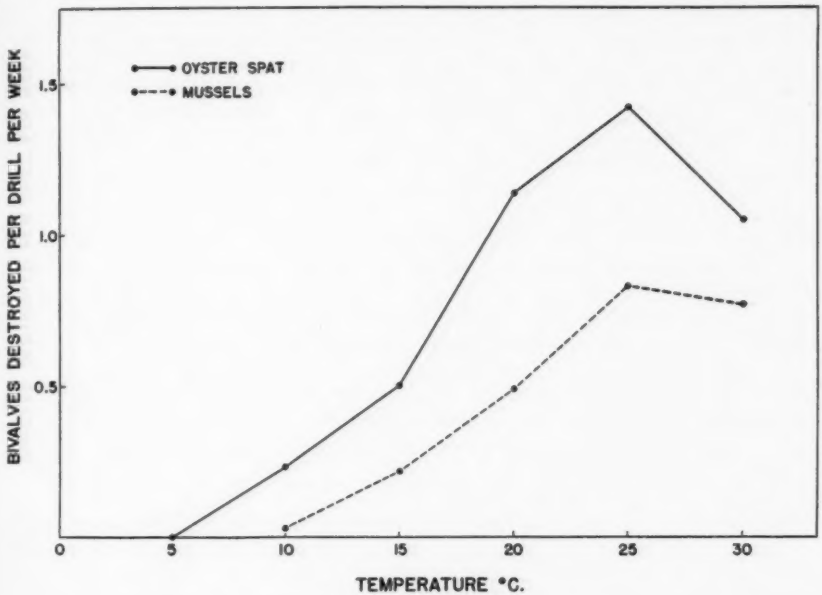

FIGURE 1. Feeding rates of *U. cinerea* at constant temperatures on young oysters or mussels. (Points are based on combined data of replicates at each temperature level.)

indicated a minimum difference between means of 0.162 for oyster spat as food. Thus, all gaps between means are significant except between 20.0° and 30.0° C. (Fig. 1). With mussels as the food organism, the minimum difference was 0.136. Thus, all gaps between means are significant except between 25.0° and 30.0° C.

DISCUSSION

The rate at which the drills destroyed oyster spat was higher than the rate at which they destroyed mussels at each of the temperatures used in these experiments (Fig. 1). This may indicate a preference of the drills for oyster spat, rather than mussels, as food. However, it is more probable that much of this difference was due to the spatial arrangement of the mussels in the tray, which necessitated more movement by the drills between feedings than when oyster spat, growing in clusters, was used for food. In addition the slightly larger average size of the mussels gave the drills somewhat more food per mussel killed than per oyster spat.

This comparison of feeding by drills on two species of bivalves shows a marked increase in destruction for each five-degree rise in water temperature within the range from 10.0° to 25.0° C. With either food, the peak of feeding occurred at 25.0° C. Since this temperature is seldom reached over the oyster beds in Long Island Sound, it is doubtful that drills in our waters are often feeding at their maximum rate.

As the drills did not feed at 5.0° C. and their rate of feeding on oyster spat was lowest at 10.0° C., a similar experiment was run at 7.5° C. to determine whether this temperature was above or below the threshold at which feeding on spat could occur. As with drills at 10.0° C., feeding at 7.5° C. was sporadic. In one experiment when 20 drills were used, one spat was killed after 27 days and two more during the next 37 days. Another group of 20 drills destroyed two to three spat per week during the first two weeks, did not feed for the next two weeks and, again, killed two to three spat per week for the final four weeks of the experiment.

The minimum temperature for feeding of drills has been reported as 9.5° C. (Galtsoff *et al.*, 1937), 9.0° C. (Loosanoff and Davis, 1950–51), 8.0° C. (Engle, 1953), and 6.5° C. by Andrews and McHugh (Carriker, 1955). The last figure was an average over a period in which the maximum temperature reached 9.5° C. and, therefore, cannot be regarded as the actual temperature at which feeding occurred. The lower temperature limit for feeding of 7.5° C. established in this study is in general agreement with these figures. However, it is apparent that short-term observations will not suffice to determine the lower temperature limit for feeding. Drills went as long as 27 days at 7.5° C. before any feeding occurred, and thus, it is possible that drills will feed at even lower temperatures, if kept for many months. In this study no feeding on oyster spat was observed at 5.0° C. during 52 days of exposure.

The author wishes to express thanks to Dr. V. L. Loosanoff for his many helpful suggestions and guidance throughout the experimental work, to Mr. C. A. Nomejko for preparation of the graph, and to Mrs. Barbara Myers for statistical treatment of the data.

Summary

1. Feeding by drills, *Urosalpinx cinerea* (Say), of Long Island Sound did not occur at 5.0° C.

2. The lower temperature limit for feeding was about 7.5° C., although feeding at this temperature was intermittent.

3. The feeding rate increased steadily as the water temperature was raised from 10.0° to 25.0° C., and decreased as the temperature was increased from 25.0° to 30.0° C.

4. The optimum temperature for feeding, when either *Crassostrea virginica* (Gmelin) or *Mytilus edulis* Linné was used as food, was 25.0° C.

5. The upper temperature limit for feeding was about 30.0° C.

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ORIENTATION IN BOX TURTLES, TERRAPENE c. CAROLINA (LINNAEUS)

EDWIN GOULD[1]

Homing ability of the box turtle, *Terrapene carolina*, was reported by Breder (1927) and by Nichols (1939), who recorded several returns to the home territory after transportation to distances as great as 4200 feet. Stickel (1950) estimated the average diameter of the range of this species to be about 350 feet, basing this estimate on 440 recoveries of 55 turtles. Home ranges of individual turtles overlap, and most recorded territories were quite similar in succeeding years. Longer journeys may be made to lay eggs or for other reasons, and some turtles had two home ranges. The longest spontaneous movement recorded was 2540 feet. But these important observations give no indication of the means of orientation employed by these turtles in finding their way home.

During the summer and fall of 1956, while stationed at the Army Chemical Center, Edgewood, Maryland, the author had the opportunity to experiment with over 100 of these turtles in an area only thirty-five miles from the location of Stickel's studies. The results demonstrate that many turtles of this species start to walk in the homeward direction within a few minutes after being released in unfamiliar territory, and furthermore, that the sun is one of the cues on which this orientation is based. These preliminary findings are of considerable interest, since they demonstrate that box turtles are capable of at least the same order of precision in celestial navigation as the birds studied by Matthews (1955) and Kramer (1953).

I am greatly indebted to the members of Boy Scout Troop 369, who brought many turtles and helped with certain field experiments which would have been impossible without them. Special thanks go to James Laney, who was particularly helpful in actively searching for turtles when they were most needed. My appreciation is also extended to Kile R. Barbehenn, Kenneth S. Rawson, and Lucille F. and William H. Stickel for their advice, to John R. Audett for drawing the figures, and to Jason I. Adleman for statistical advice. To Donald R. Griffin I am particularly indebted for his encouragement and numerous important suggestions concerning the planning of the experiments and the analysis of the resulting data.

Methods

Turtles were picked up in the fields and woodlands about Edgewood. The source of each turtle was carefully noted on a topographic map and it was given a permanent number denoted by combinations of notches filed into the edges of the posterior carapace (Cagle, 1939). In order to facilitate visual recognition in the field, its number was also painted on the shell or written with a wax pencil and covered with balsam.

[1] Present address: 475 Longview Road, South Orange, New Jersey.

When observing a turtle for a short period it was of the utmost importance that it continue its heading as long as possible rather than stopping to make a form—a well-shaped cavity made by digging with the front feet and pushing and moving about from side to side in high grass or soft soil (Stickel, 1950). Level fields having closely cut grass were found to be most suitable for release areas. In hot weather the turtles would often head for a shady spot, and release points were therefore located insofar as possible near the center of large open areas such as a parade ground or golf course. The three areas used for most of these observations are described below.

Release Point G. Part of a golf course, this area is 350 yards long running east and west and about 125 yards wide, with a northern border formed by a vine-covered fence and high trees several hundred feet in the background. Along the southern edge stands an almost unbroken line of buildings. To the east is a well-traveled road bordered by large shade trees spaced about 80 feet apart. The western portion narrows to a blunt point of a few buildings and some low shrubs.

Release Point P. A parade field, 250 by 400 yards, was found to be the most suitable release point available. Its length runs north and south; the southern end was not visible to the turtles, due to the slight elevations of the ground. A line of buildings and shrubs are to the north. Along the west side is a broken line of mixed coniferous and deciduous trees, and to the east is a road beyond which lies another group of buildings.

Release Point B is located 0.35 mile south of G, and P is 1.55 miles south-southwest of B. For the various turtles used, the distances from the release points to the home ranges varied between 0.28 and 5.80 miles; and since all release points are located within an active military post and surrounded by roads, fences, ditches, and numerous buildings irregularly situated, there was little chance that they were within territory previously visited by these turtles.

Methods of observation

Forty-three box turtles were released and observed at least once, and 22 proved to be suitable for these experiments. Selection was based on whether the turtle headed in approximately the homeward direction, and whether it moved any appreciable distance during the first period of observation, which lasted for from ten minutes to two hours. Carr (1952) mentions the various differences in the personality of turtles, and I found, for example, that some would never even open the hinges of their plastrons during a 60-minute period of observation; others would not pull themselves in unless touched, and were quite active when let alone; still others were completely at ease even when approached or picked up.

Groups of two to twelve turtles were placed at numbered stakes on the same part of one of the three fields in each release. The head of the turtle was faced opposite to the homeward direction so that a heading in the direction faced would not be confused with an accurate heading. As each turtle walked away from its stake, the compass bearing of its course was read by a compass accurate to two degrees; then the distance traveled was paced off and a piece of cloth dropped just behind the turtle. After recording one I then went to another turtle and checked its progress so that the first was not disturbed in my absence. Unless witnessed, it is difficult to imagine how little effect the observer has upon the heading of the turtle under

these conditions. It is true that some will often stop suddenly and pull into their shells when closely approached, but this seemed to have no effect whatever upon the direction in which they continued to travel. Movements over distances from 65 to 600 feet were measured, the distance depending on the time available and the activity of the individual turtle.

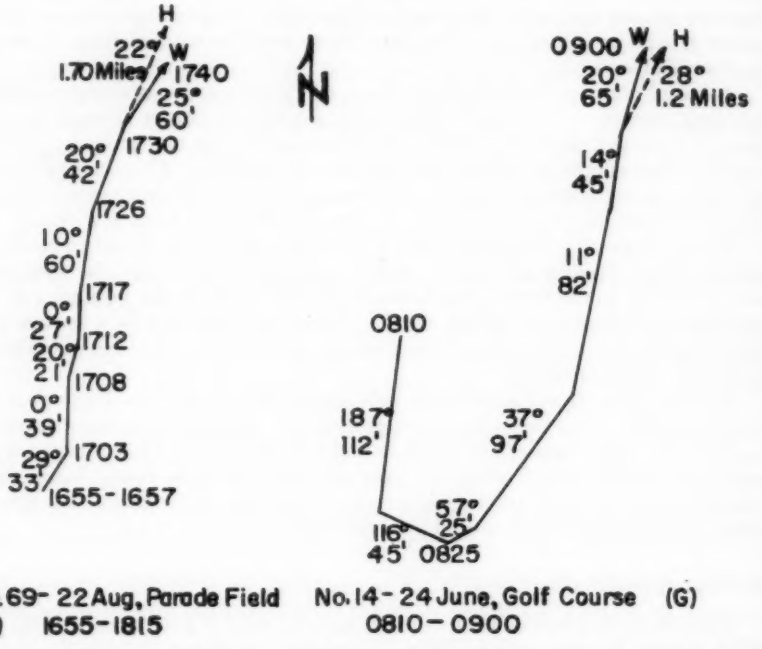


FIGURE 1. Two typical headings. H is home direction; W is direction in which the turtle went. Each straight portion of a turtle's course indicates the direction and distance traveled between compass readings. Time of day (EST) is also noted beside most of the bearings.

Because it was sometimes impossible to spend an hour watching the turtles, they were often left alone and their course recorded by attaching a spool of thread to the top of the carapace, a modification of the procedures described by Breder and Stickel. The end of the thread was fastened to a stake and unwound as the turtle walked off. Even in the short grass the slack thread would catch on small uncut weeds and the like, and would leave an accurately plotted course. A total of forty-five observations out of approximately 200 were made in this way. There were never any apparent differences noted in the headings between those recorded with and those without the use of thread. Both Breder and Stickel agree that turtles carrying the spools of thread move and behave quite normally. Stickel compared recorded movements of both and could find no discernible difference.

The turtles were transported in cloth bags or in closed boxes so that they could see nothing of their surroundings, and they were taken to the release areas by auto-

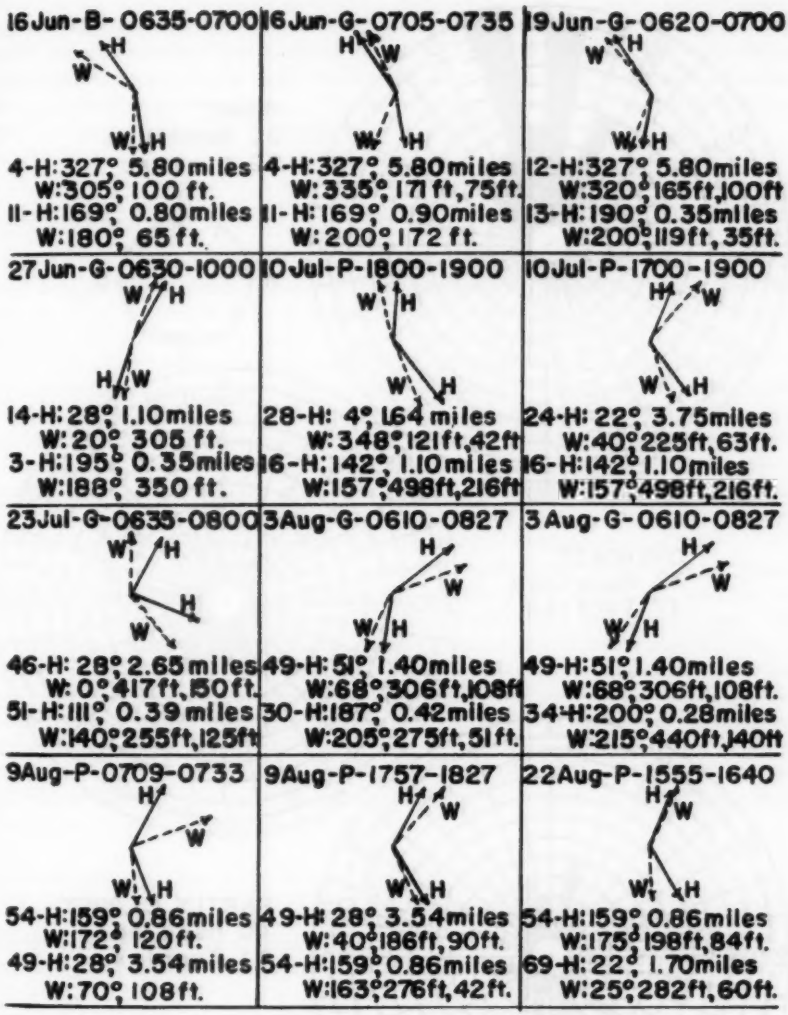
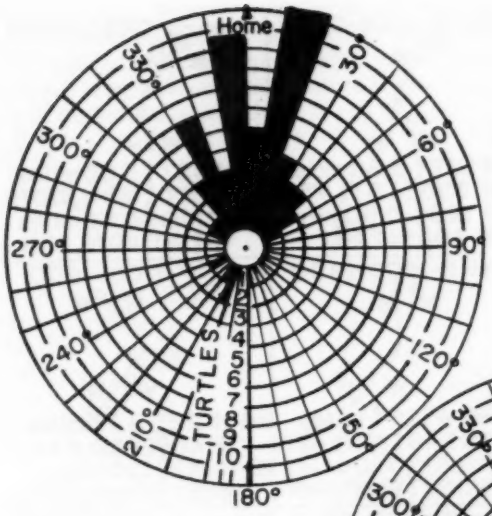
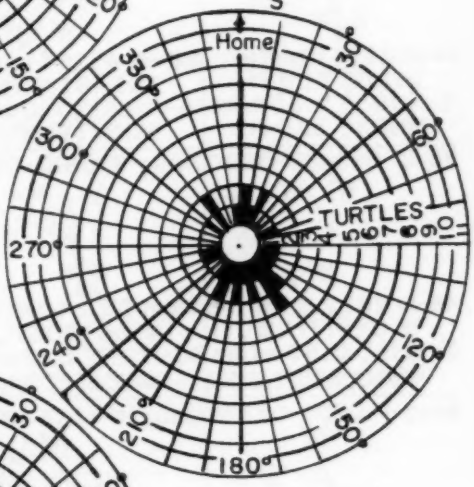


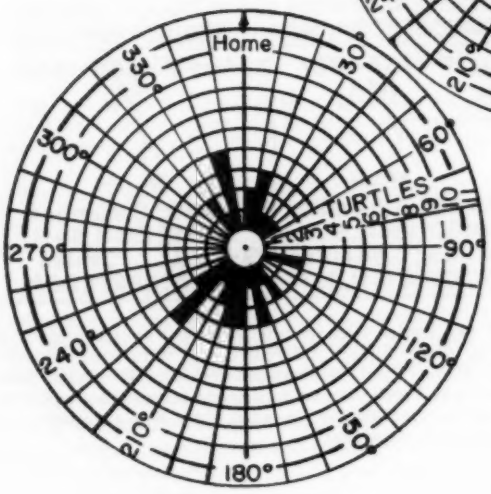
FIGURE 2. Twelve cases in which two turtles with opposite homeward directions were released from the same point at the same time. Sixteen different turtles were used. Symbols used: Release Points B, G, P. Time is EST. Turtle numbers at lower left. H is home direction and distance to home. W is direction and total distance turtle went before being picked up. Second distance indicates the length of the last direction headed if there was a variation. If only one distance is shown the course was a straight line.



SUNNY
50 Headings
22 Turtles



OVERCAST
32 Headings
17 Turtles



PARTLY CLOUDY
40 Headings
21 Turtles

mobile or bicycle. Between experiments they were kept in a small storage shed or in a pit dug in a woodlot. While in captivity they were well fed with fruits, vegetables and cockroaches, and allowed to wallow in muddy water when they pleased. Observations were usually made during the morning and late afternoon because of the objectionable effect of heat. On very hot days the turtles would immediately head for the nearest shade or would burrow into a form.

Observations

Typical behavior after release

In Figure 1 are shown two typical cases in which the headings of two turtles in clear weather were measured several times for about three-quarters of an hour after release. No. 14 started walking immediately after being set free, and No. 69 moved off after only two minutes' delay. No. 69 was returned to the same release point immediately after the heading shown in the figure, and it was observed to walk over a very similar course. No. 14 had been faced away from home, but it turned within 15 minutes and then continued in an essentially correct course until the observation was terminated. The heading chosen by a particular turtle was thus consistent from moment to moment, and was also very similar in most cases when the experiment was repeated on the same or on different days. On seven occasions a turtle was moved about 300 feet after it had indicated a definite heading, and in no case was there a significant change in the direction the turtle continued to head.

To determine whether local factors unrelated to the home direction were guiding the turtles, a total of twelve experiments were performd in which two animals from quite different home ranges were released at the same time and at the same release point (often within ten feet of each other). The turtles were so selected that the homeward direction would be 90 to 180 degrees different for the members of each such pair. If some local factor were responsible for their initial headings, both should be more or less similarly affected; if the direction of home was the important factor, they should separate. The results of these experiments are shown in Figure 2. While not all headings were perfectly correct, there is no doubt that each animal selected the approximate direction of its own home independently of the direction chosen by the other member of the pair.

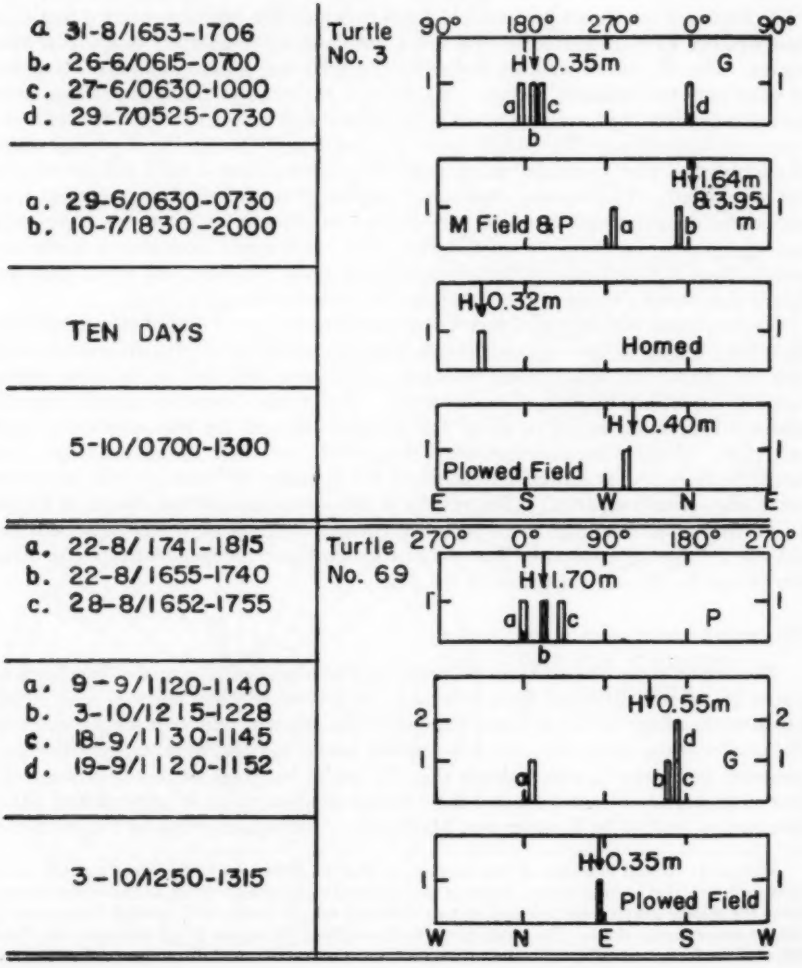
The importance of clear skies

As soon as these observations of homeward headings of box turtles had been repeated on several different days it became obvious that when the sun was clearly visible the headings were far better than when the sky was partly or wholly overcast. The results of the whole season's experiments under varying weather conditions are presented in Figure 3, which shows that the initial headings of these turtles are at least as accurately directed toward their homes as were those of pigeons and Manx shearwaters studied by Kramer and Matthews. The upper graph of Figure 3 con-

Figure 3. Initial headings of box turtles (plotted as deviations to the right and left of the homeward direction) under sunny, overcast, and partly cloudy skies. Most of the seventeen turtles in the middle graph were released at two different release points with similar homeward directions under sunny skies. The small circle to the right of the upper graph indicates the distribution of the forty-one different homeward directions which were used in sunny-weather releases.

tains the observations of twenty-two turtles which in most cases were tested at two or more release points as described above. The actual homeward directions were widely distributed around the compass. As has already been made clear in Figure 2, these headings represent in most cases a definite choice of the homeward direction.

It is of great interest that these accurate homeward headings largely disappeared under partly or completely overcast skies, for this at once indicates that the turtles were choosing the home direction by some form of celestial navigation similar to that studied in birds by Matthews, Kramer, and others. These releases were made at distances varying from 0.28 to 5.6 miles away from the place where the turtles



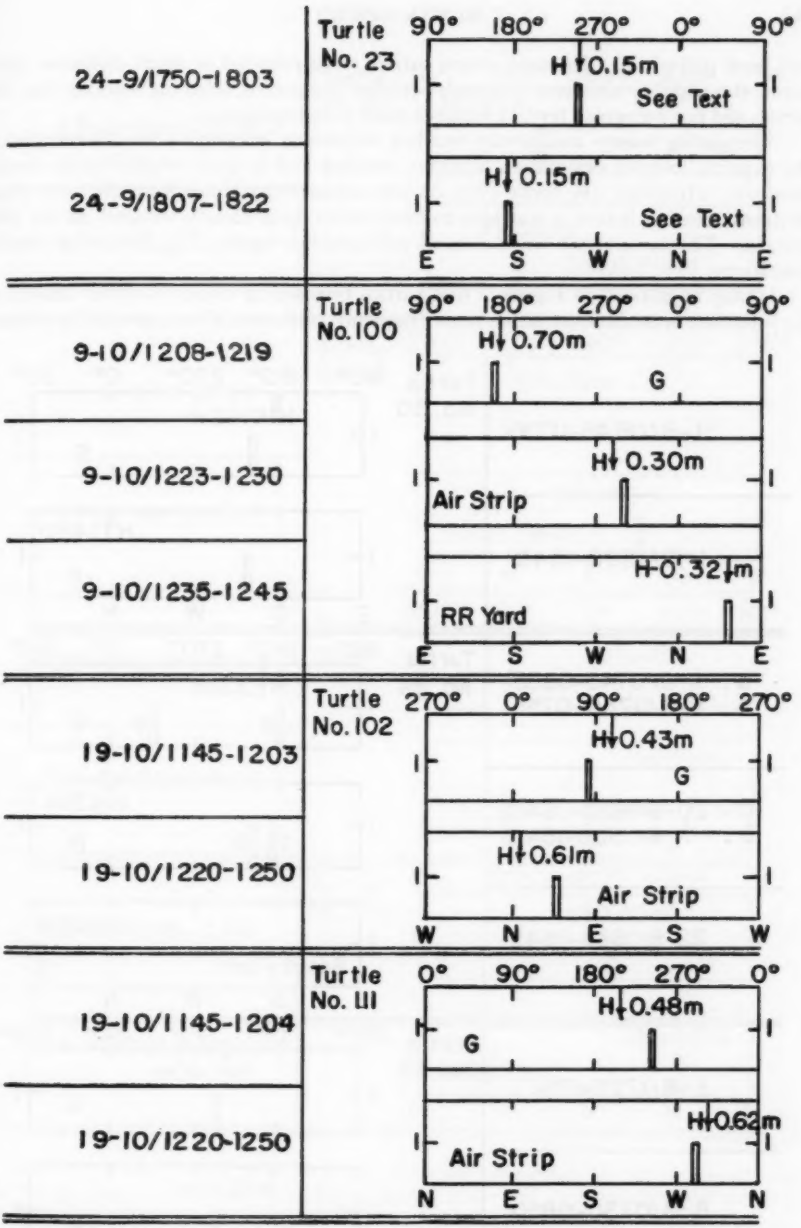
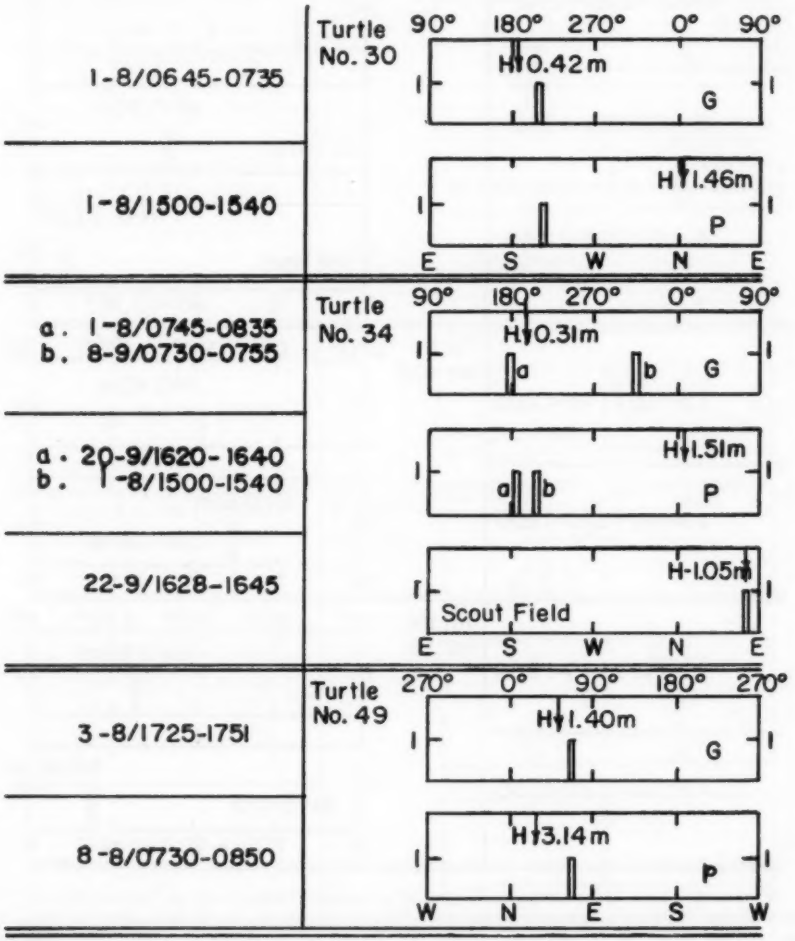


FIGURE 4. Type III orientation illustrated by graphic comparisons of turtles released from areas having different homeward directions. Release points: G, P, M field, Plowed field, RR yard and air strip. H is homeward direction. Distance from home in miles. Date and time in left column.

had been picked up. In cases where turtles were released at short distances from home, the inability to orient in cloudy weather supported other indications that the turtles did not recognize terrain features from past experience.

Alternating sunny- and cloudy-weather releases at irregular intervals resulted in the expected correct orientation in sunny weather and in poor orientation in cloudy weather. However, one turtle (No. 3) was released five times from the same place in sunny weather before it was able to head correctly in cloudy weather on the fifth release. The same result was achieved with another turtle (No. 30) under similar conditions.

It may be noticed in Figure 3 that turtles released in cloudy weather headed in the direction opposite from home more frequently than would be expected by chance.



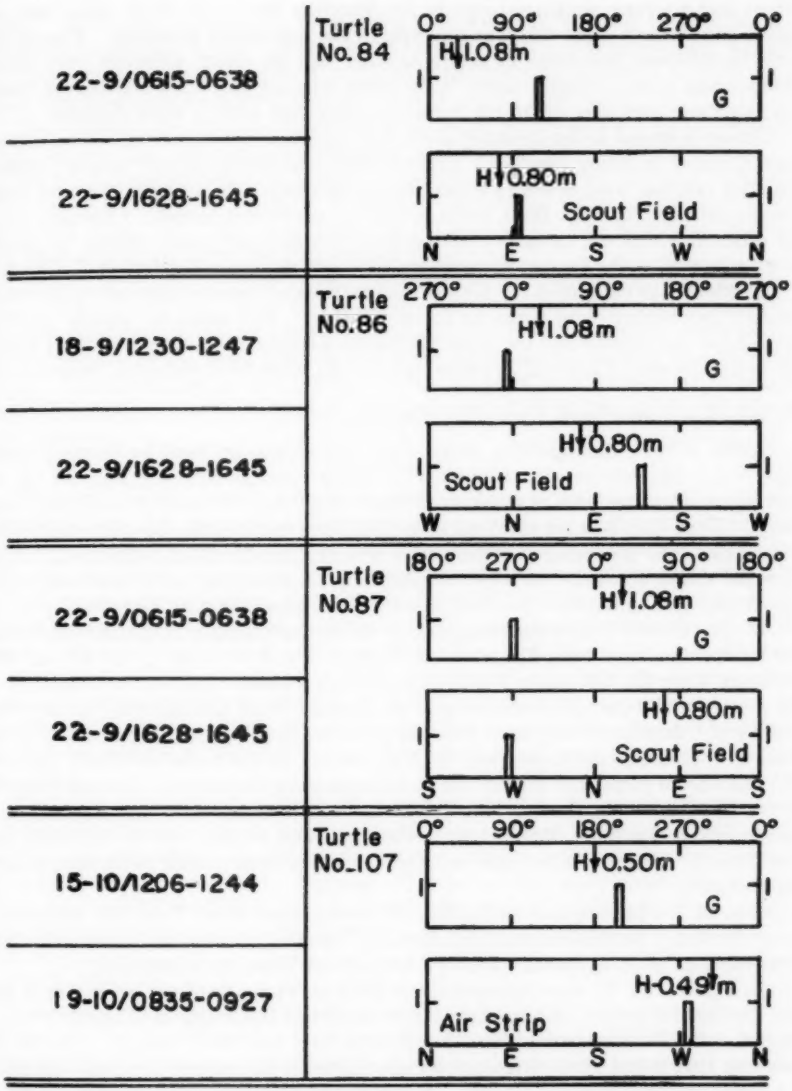


FIGURE 5. Type II orientation illustrated by graphic comparisons of turtles released from areas having different homeward directions. Release points: G, P, Scout field and air strip. H is homeward direction. Distance from home in miles. Date and time in left column.

Others had a strong tendency to go in the direction they were faced when first released, and in most cases this was opposite to the homeward direction. Facing the turtle in different directions in sunny weather had no effect, although very active turtles which move off immediately upon being released will often start in the direction faced and then veer off to the homeward direction after a short distance.

Turtles released under overcast skies showed other differences in behavior from those released in sunny weather. Eight turtles released in cloudy weather walked in circles ranging from seven to thirty feet in diameter. In a single case one turtle actually retraced its track three times in a circle seven feet across. One very obvious difference was the hesitancy of the turtle immediately after release in cloudy weather as well as the shorter distance traveled and the general inactivity. The rate of movement was computed for sixteen turtles released twenty-four times in cloudy weather and twenty-eight times in sunny weather. The mean for cloudy weather was 3.2 feet per minute and the mean for sunny weather, 5.8 feet per minute; standard deviations were 1.6 and 2.5; variations ($sd/\bar{x}$) were 50% and 43%, respectively.

Evidence for Type II and Type III orientation

Griffin (1952) distinguishes three types of orientation used by homing birds. Type I is a reliance on visual landmarks within familiar territory and the use of exploration or some form of undirected wandering when released in unfamiliar territory. Type II is homing in which an animal goes in a certain direction even when it is carried into unfamiliar territory in a new and unaccustomed direction. Type III is the ability to choose the approximately correct direction of its home even when it is carried into unfamiliar territory in a new and unaccustomed direction.

All the relevant data concerning individual turtles released from different homeward directions have been illustrated in Figures 4 and 5. Nos. 3, 23, 69 and 100 in Figure 4 are the best examples of Type III orientation. All but two headings of No. 3 were well-directed toward home. At Release Point G on 29 July an incorrect heading of 0 degrees closely resembled the previous heading, 348 degrees, at Release Point P. This appears to be Type II orientation. In the second example, M field, 3.95 miles from home, had almost the same homeward direction as Release Point P. In the third example shown, No. 3 returned to its home after ten days or less. Taking into account the rugged terrain the turtle had to traverse, the difficulty was considerable. Deep ditches, logs and dense mats of honeysuckle presented numerous obstacles.

Most of the headings of turtle No. 69 demonstrate good Type III orientation except for the 9 September record at Release Point G, which closely resembles previous releases at P, apparently another example of Type II orientation.

On 2 July, No. 23 was captured about three miles south of the pit where it was kept during the period of experimentation until the last week of August, when it escaped. On 24 September it was found only forty feet from the pit. On the assumption that it had made this place its new home, it was placed in a bag and taken to a large clearing 0.15 mile away. While walking to this release point I rotated and spun the turtle in various directions so as to remove any possibility of its remembering the movements. It was in my pocket for the remainder of the short journey. Upon release, No. 23 headed accurately toward the spot where it had just been recaptured. Following a similar procedure, it was again released from a point having a homeward direction 72 degrees different from the previous release

point. Again the heading was toward the spot where it had just been recaptured. It started in the direction it had proceeded from the previous release point, but after a short distance it headed correctly. Neither the previous direction nor the homeward direction corresponded to the direction the turtle was faced.

No. 100 was released from three different release points within a period of thirty-seven minutes; all headings are supportive evidence for Type III orientation.

Figure 5 is a compilation of records of turtles released from different homeward directions which showed evidence of Type II orientation or of an ambiguous type which may have been Type II or Type III. The others in this figure are less obvious but complete the collected data on this subject.

Another instance of Type II orientation was demonstrated by ten releases of eight turtles from New Jersey and Massachusetts, 180 and 400 miles, respectively, from the home grounds. In six out of ten cases the headings closely resembled the same directions taken when last released from points approximately one mile from home. The differences between directions headed from New Jersey and Massachusetts release points and those at close distances were 0, 3, 3, 10, 14, 17, 38, 43, 148, 155 degrees.

Discussion

While the turtle is moving on its course the head is held high and is directed forward so that if it is walking in a direction opposite to the sun's position it is necessary for the turtle to turn its head in order to see the sun directly. This very thing was frequently observed. The turtles would stop, look about them for thirty seconds to a minute or more, and then continue on their way. These stops were often made every two to four minutes, varying considerably with the individual turtle. Many turtles showed ability to maintain a straight course. One, for example, traveled 200 feet and kept within three degrees of a straight line for twelve minutes. Seven turtles released before sunrise moved but little until the sun was visible. Turtles released just before sunset either stopped after the sun had set or changed to a wrong direction after having followed the correct course while the sun was visible.

Twelve turtles were recovered after escape or release from a pit where they had been in captivity. Four turtles actually homed from distances of 405 to 563 yards. One returned to the pit from another release point after having been in captivity in the pit most of the summer. The remainder were picked up at points which were nearly in direct line with the homeward direction from their starting point. One of these had traveled 458 yards in less than six days in a presumed line which was only nine degrees from the homeward direction. The distance to home was 4.47 miles.

In some preliminary experiments turtle headings were recorded under natural sunlight and then observed in the shadow of a person or tree when the sun was reflected onto them from a mirror placed about 180 degrees from the sun's position. In all of twenty observations using ten turtles they changed their courses significantly when the sun's image fell upon them. In nearly all cases the turtles headed toward the mirror. Furthermore, in all cases the headings which were initially taken in the sun were resumed after the mirror was removed and the turtle was returned to natural conditions of direct sunlight. The exact meaning of these observations remains obscure, but they do support the hypothesis that it is the sun which enables these turtles to exhibit Type II and Type III orientation.

Summary

1. An investigation was made to test the hypothesis that box turtles [*Terrapene c. carolina* (Linnaeus)] employ a means of sun orientation similar to that found in birds. Box turtles from different localities were taken in closed containers to unfamiliar territory and released in large open fields 0.28 to 5.80 miles from their homes. (According to Stickel the home range is about 300 feet in diameter.) They were then observed over periods varying from ten minutes to two hours, and with the aid of a compass their headings during that period were plotted, and the distances traveled were paced off. After the observation they were again placed in closed containers and returned to a pit where they were kept until the next release.

2. Of forty-three turtles released and observed in this manner, twenty-two headed toward home; seventeen of the latter were released under sunny and overcast skies and in most cases this was done from at least two different release areas. Homeward headings were observed in sunny weather, but under overcast skies orientation broke down (Fig. 3). Twelve examples, two turtles each, of situations in which sixteen turtles with opposite homeward directions headed toward their respective homes at the same time from the same place, demonstrated that the heading was not dependent on some local environmental factor at the release point (Fig. 2).

3. Of sixteen turtles released from several completely different homeward directions, four showed definite ability to orient correctly (Fig. 4).

4. There were ten releases under sunny skies more than 150 miles from home. Nine turtles were used in these experiments. In seven of these headings the turtles went in a direction which seemed to correspond to the direction last chosen when close to home, regardless of the actual homeward direction. At short distances of a mile or less from home several turtles also headed in consistent directions regardless of the homeward direction.

5. Ten turtles in twenty experiments were first observed for directional heading in natural sunlight, and then observed in the shade while the sun's image was reflected upon them with a mirror. The direction of heading was altered in all cases and usually resulted in the turtles' heading for the mirror.

6. While walking, turtles stopped frequently and turned their heads as though looking at the sun.

7. These findings seem to support the hypothesis that turtle orientation resembles the type of orientation found in birds; however, more data are necessary to clarify numerous factors which may bring to light conflicting differences and close resemblances. Work is continuing and new developments will be reported as they are observed.

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AN EXAMPLE OF REVERSAL OF POLARITY DURING ASEXUAL REPRODUCTION OF A HYDROID

CADET HAND AND MEREDITH L. JONES

Department of Zoology, University of California, Berkeley, California

A number of small and curious hydroids have been described over the years. These hydroids, in general, occur on soft bottoms as members of the fauna of the upper few millimeters and are of rather diverse asexual reproductive habits. This group, most of which are unrelated systematically, includes *Boreohydra, Psammohydra, Corymorpha, Heteractis, Euphysa* (the last three closely related), *Halermita* and *Microhydra*. In view of the apparently wide distribution of this type of hydroid in soft bottom communities it is probably not surprising that still another form has been discovered in San Francisco Bay, California.

On November 28, 1954, we recovered two live specimens of such a hydroid from a core sample 18 mm. in diameter taken at a depth of 30 feet off Pt. Richmond. The bottom at that location consists of grey mud overlain by about 5 mm. of loose debris, from which the hydroid to be described was taken. According to Sumner *et al.* (1914) the annual salinity in this area ranges from 18.0‰ to 32.3‰ with a mean of 27.0‰.

Morphology and Reproductive Behavior

The two specimens were a single polyp and a pair of polyps united at their base. Each had a small patch of debris adherent to its base. Figure 1 illustrates the pair. The polyps were quite extensible and were about 1–1.5 mm. long when maximally extended. The hydrocaulus was narrowest at the base and gradually enlarged toward the area of tentacular insertion, where the diameter was approximately twice that of the more proximal region. The tentacles were filiform and were capable of extending to about 1 mm. They were inserted in a single cycle at the base of the proboscis. The number of tentacles varied from as few as 4 to as many as 12, although, of the original specimens, the solitary individual had 6 and the pair had 9 and 10 at the time they were first observed. Due to the extreme contractility and extensibility of the hydroid, the relative length of the proboscis and the hydrocaulus was quite variable. In general, however, this relationship varied from 1 : 1 to 1 : 3 (*i.e.*, with the hydrocaulus about three times the length of the proboscis, at times). The color of the hydroids was a light, flesh-pink, and the whole animal was translucent.

The two original specimens were set aside in a stender dish to which was added a small amount of debris from the core sample. The specimens were observed at least once a week following this and were fed the nauplii of brine shrimp (*Artemia*) at each observation. Early in January it was noted that there were two pairs of polyps plus the single one in the culture dish. At the time it was assumed that the extra pair had been overlooked at the time of isolation. However, on January 24,

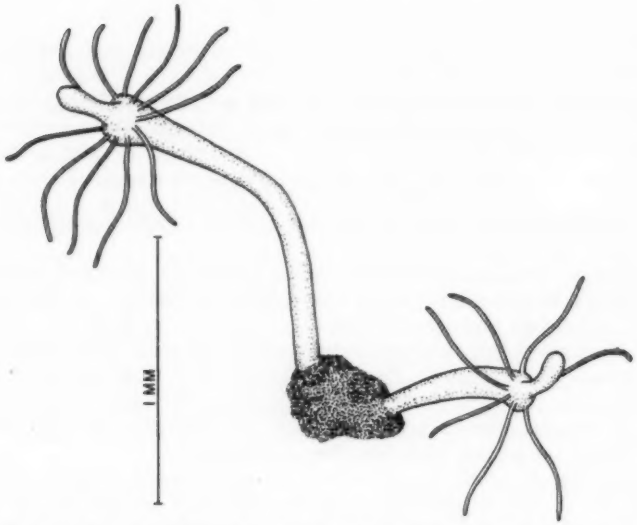

FIGURE 1. The original paired hydranths. The individual on the left has elongated preparatory to undergoing asexual division.

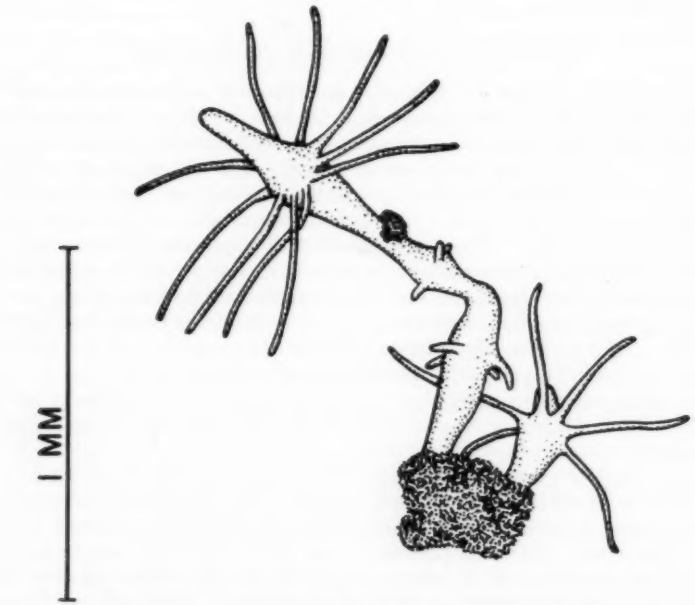

FIGURE 2. A polyp pair in process of asexual division.

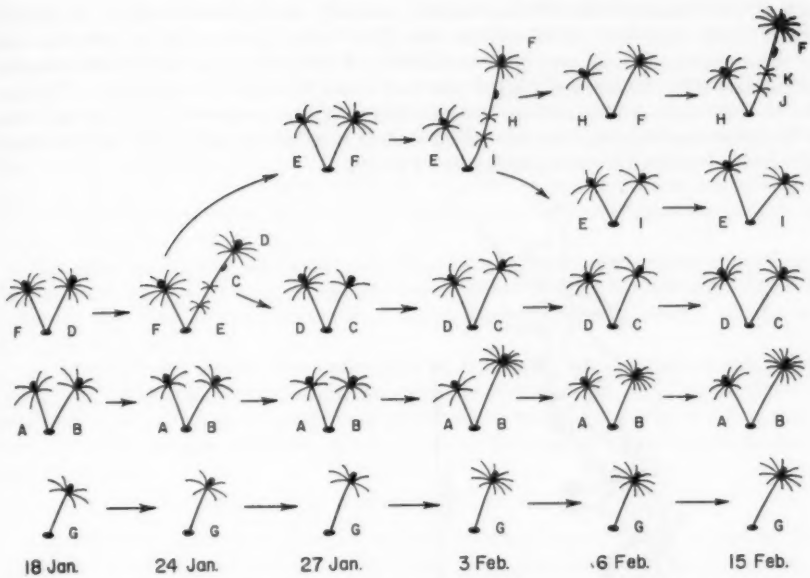

FIGURE 3. A schematic diagram showing results of observations on critical dates from January 18 to February 15, 1955.

1955, it was observed that a polyp of one of the pairs had become about twice the length of its partner and possessed two additional sets of tentacles, intercalated between the base and the original whorl of tentacles, and further, at a point between the new tentacles and old hydranth, debris had collected and was adherent to the hydrocaulus (Fig. 2). Daily observations following this made it clear that we were witnessing an unusual mode of asexual reproduction which resulted in the production of pairs of polyps. By January 27, fission had occurred between the two new sets of tentacles, and the area of adherent debris had become the base of the new polyp pair. Pursuant to this discovery we began a series of daily observations extending from January 24 to February 15.

For convenience in record-keeping, letters were assigned to the polyps (these letters will be referred to in the text, where necessary), and the accompanying figure (Fig. 3) illustrates, in a diagrammatic fashion, the results of our observations from January 18 to February 15. At this time there were four pairs and a single polyp. The polyps "F" and "D" were the only polyps to divide during this period, and one of these probably gave rise to polyp pair "A-B." In each instance the elapsed time between the first appearance of new tentacles and actual divisions was three days.

Following February 15 our observations became somewhat irregular, and a single observation on March 4 revealed that we now had seven pairs of polyps and that two of the pairs were preparing to divide once more (the single specimen had been sacrificed previously for nematocyst data). Immediately following this observation, the dish containing the hydroids became badly fouled (presumably as a re-

sult of over-feeding with *Artemia* larvae, and our subsequent failure to change the water), and on March 8, when this was discovered, the remaining hydroids were in what appeared to be very poor condition. Each polyp was tightly contracted to about one-third its normal height and was about 0.5 mm. in diameter. The tentacles were mere knobs and the animals had assumed a nearly hemispherical form. The water was changed, but this failed to save most of the specimens and by March 15 we were left with but a single pair of polyps.

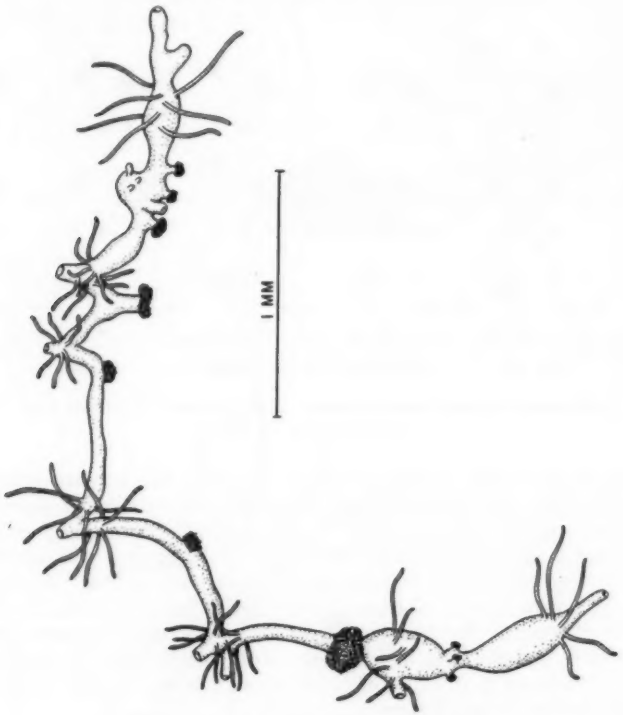

FIGURE 4. The monstrosity on March 25.

On March 21 we observed that our remaining pair had once more produced two new polyps, but although these were well-formed, they still had not separated. On the next day (March 22), although the two new polyps had not yet parted, there were signs that still another pair of polyps was being formed, giving us six polyps in series, and this specimen was apparently becoming a monstrosity. We also noted that near the middle of the central hydrocaulus there was a single protuberance and a group of four structures which looked like still another pair of incipient polyps with irregularly placed tentacles. By the next day (March 23) our specimen had opened another mouth between a pair of orally-directed polyps and we could now count 12 sets of tentacles and 4 open mouths. By March 25 the animal had grown

to about 4.5 mm. long (Fig. 4), was composed of 14 recognizable individuals and had 8 mouths which seemed to be functional (in that they were seen to open and close and brine shrimp larvae were ingested by several).

On March 28 the specimen had finally succeeded in dividing into pieces, and we were able to count two single polyps, two separate double polyps, one triple polyp (three mouths and sets of tentacles with a single base), one quadruple (four mouths, three sets of tentacles and a base), and two groups of polyps which had three and four mouths each. These last two groups consisted of rather curious assemblages of mouths, misplaced tentacles, and bits of adherent debris (suggesting bases) at irregular points over the masses.

This situation, that is, with eight variously assorted hydroids ranging from single individuals to groups of four, was maintained without further change until April 4. Following this date, the numbers were reduced by death, and by June 1, all the individuals had died.

As stated above, during the earlier part of our study, the original single polyp (polyp "G," Fig. 3) was sacrificed to study its nematocysts, and, unfortunately, none of the polyps or polyp pairs was preserved. Although we have taken more than 1200 core samples from the general area of the original collection, none have been found since.

Description of Asexual Reproduction

The sequence of events leading to the production of new pairs of polyps in this hydroid has been closely observed. The earliest sign that the process was underway

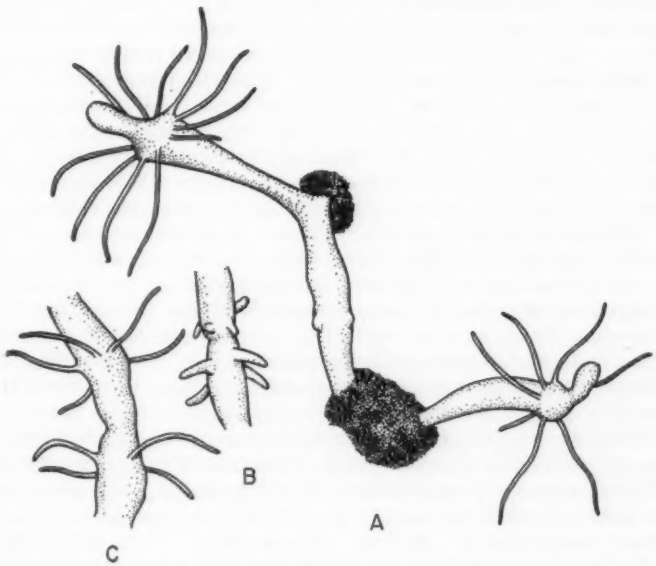

Figure 5. Development of tentacles prior to asexual division. A, the polyp pair with new tentacles barely recognizable (February 14); B, tentacle growth in 23 hours (February 15); C, tentacle growth in 40 hours (February 16).

was the adherence of a small bit of debris to the hydrocaulus at about its mid-point, although this was commonly nearer the tentacles than the base (Fig. 5, A). This phenomenon was associated with a slight increase in the opacity of the tissues at this point, and the protrusion of a small knob of tissue. This knob within 24 hours of its appearance became completely obscured by adherent debris. In addition, during this first phase of development, two sets of minute protuberances were seen in the mid-region of the hydrocaulus, between the adherent debris and the original base (Fig. 5, A). Within 24 hours, the protuberances grew into recognizable tentacles, usually three or four per whorl (Fig. 5, B). With the appearance of the tentacles, there is a marked increase in the opacity of the tissue in this area.

During the second 24-hour period, a translucent region developed in the area between the two new sets of tentacles, and subsequently, there was an indenting of the surface tissue of the hydrocaulus at this point. On the third day, there was a further continuation of the constriction of this area, leading to the actual separation of the two new polyps. This point of fission marked the distal end of the proboscis of each of the two new polyps, and each polyp possessed a functional mouth as soon as fission was completed; in addition, the point of adherent debris had now become the base of the newly released pair. The result of this process of fission was two pairs of polyps, each with one member (the older) about one-third larger than the new polyp.

Discussion

Many well-known studies have established the fact that hydroids possess gradients, particularly those of regenerative ability, which are more marked or rapid in more distal pieces of stems. Steinberg (1954), in studies on *Tubularia,* found that short pieces of stem (1–2 mm. long) regenerate bipolar or partial bipolar hydranths, while in longer pieces (2–12 mm.) a new hydranth is usually formed only at the original distal end of the stem, and, in still longer pieces (12–30 mm.), each end regenerates a hydranth. Steinberg also describes movements of cells toward the distal ends of cut stems and states that these cell movements precede the process of regeneration. In the hydroid described above, since the whole animal is less than 2 mm. long, one wonders if there can be effective or actual gradients such as those obviously present in larger hydroids. However, numerous studies on *Hydra,* an equally small organism, have shown that well-developed gradients also exist in small hydroids. In the animal described here, the appearance of two new orally-directed hydroids interposed in series between an extant base and hydranth must mean that a re-organization, involving a reversal of the original polarity of the hydrocaulus, has occurred, and it would seem that information obtained from experimental studies already completed on other hydroids should help to explain this observed event.

It should be pointed out that the reversal of polarity, which we assume to be present during the course of asexual reproduction, is a unique event. Hyman (1940, pp. 487–492) has reviewed much of the literature to that date relative to asexual reproduction and regulative processes in hydroids. She points out that in animals as small as hydras the polarity is well-developed and can only be altered by rather drastic treatments, *i.e.,* electrical currents, burial of the apical end of a cut stem, or by treatment with cyanides, anesthetics, and other depressing chemicals. Further, it is well known that pieces of hydroid stems and hydras retain their original polarity whether free or grafted to other pieces. We assume, therefore, that our

animal must also have a definite basal-oral polarity; that in the course of asexual reproduction by this animal, the polarity is reversed, at least for a short part of the hydrocaulus; and that this is a naturally-occurring and unusual event.

After considering all the evidence available to us, we have come to the conclusion that we can explain our observations on this hydroid if we are allowed to make one hypothesis. This hypothesis is concerned with the first event we observed during the course of asexual reproduction (*i.e.*, adherence of debris to the hydrocaulus) and is unique to the extent that it gives certain unusual features to the presumptive base of the new polyp pair. We suggest that the appearance or formation of the new base, as the first step in asexual reproduction here, must represent an effective block in the existing gradient, and that this new base must compete (in a certain sense) with the old base for the hydrocaulus between them. Since it is well-established that distal portions of hydroid stems form new hydranths, we can now consider that the portion of the stem between the bases is distal to each base and that it proceeds to develop two new orally-directed polyps simultaneously. In other words, we believe that the two bases with the common piece of hydrocaulus between them, are, individually, behaving in the same fashion as would pieces of decapitate stem, *i.e.*, that there will be regeneration of a new hydranth at the distal extremity. This, of course, must involve a reversal of the preëxisting gradient in the piece of hydrocaulus closest to the newly formed base, and we might postulate that this is the result of some disorganizational or re-organizational power of the base. To the extent that we observed a condensation or increasing opacity in the tissues at the points of formation of the two new polyps, our observations seem to fit the known facts, such as those presented by Steinberg (1954). If, therefore, our hypothesis is acceptable, we can interpret asexual reproduction in this hydroid as a phenomenon involving regeneration at distal extremities.

Cases of reversal of polarity do not seem to have been reported in nature for any other hydroids, unless we can interpret the study of Rees (1937) on *Heterostephanus* (now *Heteractis aurata* according to Kramp, 1949) as such. In *H. aurata* the polyp bud develops from the side of a hydranth, usually a tentacle base, in such a fashion that the free end of the bud is the future hydrocaulus. This same phenomenon has been observed by us in a similar hydroid (*Euphysa* sp.) from San Francisco Bay. In all other cases where transverse fission occurs, a new polyp develops at the distal end of the remaining hydrocaulus, and the new free individual develops a base at the proximal end of its own hydrocaulus. In one genus, *Psammohydra*, Schulz (1950) has found that the new hydranth develops tentacles before fission actually takes place.

Recently Kinne (1956) has made observations on *Cordylophora caspia*, an athecate hydroid from fresh and brackish water. He noted that in approximately 20% of polyps adapted to a salinity of 24‰, there were various abnormalities developed. These consisted of: the formation of intercalary hydranths on a hydrocaulus ("uniaxial hydranth aggregates"); fusion of polyps ("multiaxial hydranth aggregates"); the formation of large coenenchymal bodies, which became detached from the colony and formed new hydranths; and "globular hydranth complexes," with no stolons and no stalks.

The formation of the intercalary polyps is remarkably similar to the phenomenon observed in the hydroids from San Francisco Bay. However, there are points of difference, for in *Cordylophora*, the two new polyps are joined by the sides of the

hypostome and the mouths are directed laterally, the original polyp is a member of a colony, and there is no interruption of the hydrocaulus between the old and new polyp head which might correspond to the new base of our local forms. Kinne mentions that when the hydranth groups are detached they frequently become established on the substrate and sprout new stalks, but no intimation is made that these persist as pairs, nor that further intercalary hydranths are formed.

In an early section of this report we described a monstrosity which developed from a pair of polyps following their recovery from a period of depression. Hyman (1940) has reviewed the general effects of depression on hydroids, and notes that after recovery from depression, hydras often regenerate doubled parts or other anomalies, and that this is followed by fission processes. Kinne explains his various anomalous forms as the results of disturbances of growth and differentiation processes and a consideration of our observations leads us to evaluate our monstrosities in the same light.

However, since we noted some six apparently normal divisions, culminating in seven polyp pairs, and since the original pair was taken in the field and had not, so far as we know, been exposed to abnormal environmental conditions, we reserve judgment as to whether or not this process is abnormal, for this organism. The possibility does exist that the unknown hydroid, upon which we based our observations, is actually a *Cordylophora*, for this genus has been collected at the Carquinez Straits, approximately 15 miles up the San Francisco Bay Estuary from Point Richmond.

Systematics

We have little concrete information to guide us in the identification of the hydroid we have described. Our study of the nematocysts revealed that it possesses a simple cnidom of desmonemes and euryteles, as follows:

Small desmonemes (common)	4–5 × 3–4 μ
Large desmonemes (rare)	9–10 × 5–4 μ
Microbasic euryteles (common)	9–11 × 3–4 μ

This cnidom suggests that the hydroid is a member of the gymnoblasts (anthomedusae) or limnomedusae (see Weill, 1934; Russell, 1938; Hand, 1954). The complete absence of perisarc also suggests that the hydroid may be a limnomedusan form. However, the perisarc apparently may be completely absent in some gymnoblasts and calyptoblasts. We also know that differences may occur between the cnidom of a hydroid and that of its medusa (Hand, 1954), but in this hydroid, we do not know what medusa, if any, is involved in the life history. Therefore, we can not place this hydroid in any order with certainty, although it seems clear that it can not be a calyptoblast.

Because of the proximity of a source of *Cordylophora*, the possibility of the unknown form's being an abberant type of *Cordylophora* was explored. The main points of difference are that *Cordylophora* grows in extensive colonies, its tentacles are scattered, and it possesses a well-developed perisarc. None of these characters applies to the form described here. In addition, Hand and Gwilliam (1951) reported only a single size-class of desmonemes for *Cordylophora*, while we have found two size-groups. Thus, it seems doubtful that this is actually a growth variant of *Cordylophora*.

Since so little can be done with the hydroid from the standpoint of systematics, we will refrain from assigning it the status of a new species, pending its further collection and a more exact taxonomic determination.

The authors wish to acknowledge the assistance of Mrs. Emily Reid, to whom we are indebted for the illustrations accompanying this report.

Summary

1. Asexual reproduction, involving reversal of the original oral-basal polarity, is described.

2. Asexual reproduction in this hydroid leads to the production of pairs of polyps sharing a common base.

3. The systematic position of the hydroid is not established, although the cnidom suggests possible affinities among the gymnoblasts.

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THE ELECTROCARDIOGRAM OF A STOMATOPOD

HIROSHI IRISAWA AND AYA FUNAISHI IRISAWA

Department of Physiology, School of Medicine, Hiroshima University, Hiroshima, Japan

It is generally accepted that the crustacean heart beat is of neurogenic origin. This has been concluded from pharmacological (Krijgsman, 1952; Prosser, 1942), electrophysiological (Prosser, 1950; Hagiwara and Bullock, 1956) and anatomical evidence (Alexandrowicz, 1932, 1934). Both *Limulus* and the lobster have been extensively studied, but relatively few studies of other species of crustaceans have appeared in the literature.

Alexandrowicz (1934) has described the innervation of the heart of stomatopods, and showed that there are nerve cell bodies in the elongated ganglionic trunk, which he considers as an automatic apparatus which rules the heart beat. Since the ganglia of the stomatopod are of simpler structure than those of *Limulus*, a physiological study of the heart of the mantis shrimp may be of considerable assistance in explaining the origin of the heart beat of the arthropod neurogenic heart. This paper describes the electrocardiogram of the mantis shrimp and the detection and localization of the pacemakers of this heart.

Materials and Methods

Marine mantis shrimps (*Squilla oratoria* de Haan, Crustacea, Malacostraca, Stomatopoda) were used throughout the present study. They were fixed on a cork board, the shell opened dorsally and the preparations placed in a plastic dish filled with sea water. The dorsal muscles were dissected and removed to expose the long segmented tubiform heart which extends from the posterior to the thoracic part of the body cavity. Since the heart is closely attached to the digestive tract the experiments were carried out without isolation of the heart from the body. The preparations were decapitated at the level of the fifth segment, to avoid central nervous effects and suppress muscle activity.

The indifferent electrode was placed remotely from the heart in the sea water, while the recording electrode was moved with the aid of a micromanipulator and microscope along the median line of the exposed heart. Low resistance microelectrodes (tip diameter 10–15 μ), originally described by Tomita and Funaishi (1952), were used. The surface action potentials were amplified with a condenser-coupled amplifier, displayed on an oscilloscope and photographically recorded. When longer recordings were needed a smoked paper electrometer (Hatakeyama, 1954) was employed.

Results

Structure of the heart. The heart consists of fourteen segments each of which has a single nerve cell, a pair of ostia and arteries. Except at the proximal and

distal ends of the heart tube, the single nerve cells of each segment lie individually in the median nerve trunk behind the ostial orifices. Alexandrowicz (1934) previously pointed out that the size of the nerve cell varies with the segment. In this study, on the basis of thirteen preparations, it was found that the thirteenth segment always contained the largest cell (average value: length 80 μ, width 63 μ). There is a progressive decrease in the size of the nerve cells anterior and posterior to the cell of the thirteenth segment.

The major components of the electrocardiograms. When the recording electrode was placed on the surface of the peripheral part of the heart, relatively slow triphasic action potentials were observed as shown in Figure 1 A. As can be seen

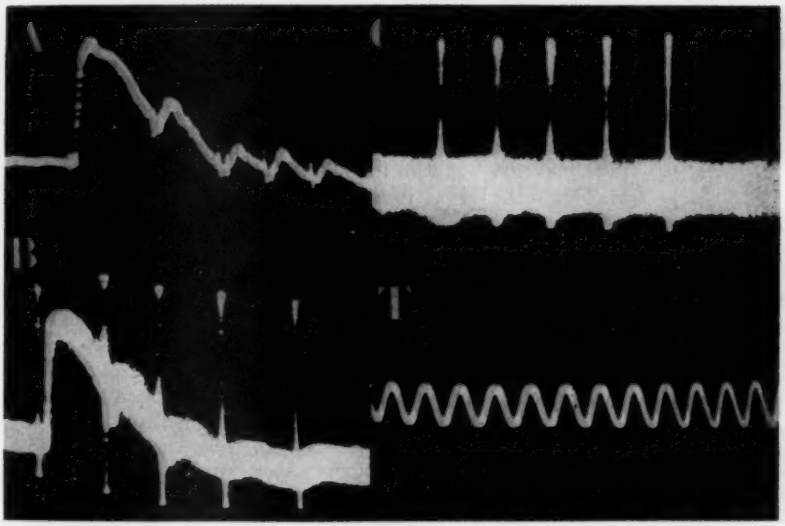

FIGURE 1. Surface action potentials of the heart of the mantis shrimp. A. Record obtained at some distance from the nervous trunk. Small rhythmic waves and small spikes can be seen. B. Record from the central part of the heart tube, characterized by a train of spike potentials and the slow muscle potentials. C. When the contraction of the heart is small or absent, only a train of spike potentials is recorded. T. Time: 60 cycles per second.

in this figure, the action potential usually reached its summit within 8 msec., and thereafter exhibited a slow exponential decay. However, when the recording electrode was placed on the mid-dorsal region of the heart, where the ganglionic trunks are located, a train of very rapid spike potentials was superimposed on the slow wave (Fig. 1 B). When the contraction of the heart tube was small or absent, only the spike train was recorded (Fig. 1 C). The spike components as observed in Figure 1, B and C had extremely brief durations not exceeding one millisecond. The height of these spikes remained relatively constant in the same preparation. However, attenuation of spikes was observed when the electrode was moved away from the median nervous trunks, as shown in Figure 1 A. The number of spikes in each heart beat varied widely from preparation to preparation, ranging from one

to about sixteen and averaging 7.2 spikes per heart beat in twenty-eight preparations. The number of spikes seemed to decrease with the duration of each heart beat.

The findings are compatible with the concept that the spike component arises from a stimulating discharge of the large single nerve cell of the heart segment and the slow potential from the heart muscle.

Location of pacemaker. Transection is a valuable method for the detection of pacemakers in this type of heart because of its segmentation. When a part of the heart muscle was dissected, no remarkable change in action potentials or heart rate was observed; however, when the nervous trunk was incised, definite changes occurred which varied according to the place of transection. It was not possible, however, to transect the nerve trunk without injuring part of the heart muscle.

TABLE I

The influence of successive isolating transverse sections upon the rate of contraction of the segments so isolated

Preparation number	Rate of heart before sectioning*	Rates of various isolated segments after sectioning anterior to segment thirteen			
		Segment number			
		13	12	11	10
1	84	84	52	30	18
2	96	96	42	16	†
3	48	48	42	18	†
4	60	60	24	†	†
5	60	60	30	†	†
6	84	84	48	†	†
7	54	54	30	14	†

* Figures in the table are rates in contractions per minute.

† Contractions stopped.

Sections were made serially between successive segments starting with a cut between numbers 12 and 13. Following the cut, a recording of the rate was made and the process repeated with the next segment.

Transection of the heart nerve trunk, starting with a cut anterior to segment thirteen, showed that the heart rate was reduced in sections anterior to the cut. The results of seven experiments in transection at successive levels are given in Table I. As can be seen from the table, the rate of beating of segment thirteen was unchanged by a section anterior to it but segments anterior to the cut beat at a slower rate. In terms of the rate of the thirteenth segment as 100 per cent, the first cut between segments twelve and thirteen reduced the rate of segment twelve to an average of 56 per cent. The next cut between segments eleven and twelve stopped the beating in three out of seven cases, but in the four that continued to beat the rate was reduced to 29 per cent of the original. The next cut stopped beating in all but one preparation.

These results clearly demonstrate that the thirteenth segment, which has the largest median nerve cell, has the fastest rate and governs the rate of the heart beat.

Discussion

The electrocardiogram of the Stomatopoda resembles that of *Limulus* (Prosser, 1950) and *Astacus* (Hoffman, 1912). However, the electrocardiogram of the mantis shrimp differs in that it consists of two clearly distinguishable component potentials: one of muscle and one of nerve. Since the ganglionic trunk in Stomatopoda lies on the surface of the heart tube, pure nervous spikes are obtained.

Prosser (1943) demonstrated bursts of impulses from the isolated *Limulus* heart ganglion. Welsh and Maynard (1951), and Maynard (1955) studied the cardiac ganglion of the lobster and showed that a burst of nerve impulses preceded and accompanied the early part of the mechanical response. Matsui (1955) found that the number of spikes within one heart beat varied extensively from preparation to preparation, ranging from several to eighty. Maynard (1955) distinguished two different spike potentials, namely, small and large components in his records. The type of spike-train which was obtained from a median ganglionic trunk of this stomatopod seems to be simpler than any other pattern of impulses obtained through the surface electrode from other crustaceans, and only comparable with those records from a single nerve cell of lobster (Hagiwara and Bullock, 1955; Watanabe, personal communication). Thus, the uniformity and the simplicity of this record seemed to be due to the simple structure of the ganglionic trunk: this was confirmed histologically (Irisawa and Irisawa, 1956).

Since the heart ganglion frequency is unchanged after the removal of the influence of the regulator nerve, the ordinary heart rate is probably regulated by the activity of the chain of ganglionic trunks. Possibly there is a dominating cell in the ganglionic trunk that synchronizes heart activity. Maynard (1955) stated that both large and small cells are the pacemaker cells; Hagiwara and Bullock (1955) suggested that the posterior small cells may be the pacemaker cells.

Our experiment demonstrated a remarkable gradient of the size of these heart ganglion cells and of the frequency of spontaneous firing by the respective segments when isolated from faster segments. Studies have also revealed variation in the sensitivity to a stimulus (Irisawa and Irisawa, 1956). In summation, the large cell of the thirteenth segment is the largest, most sensitive to a localized thermostimulus (Irisawa and Irisawa, unpublished data) and its segment has the highest automaticity. It is probable that this large cell of the thirteenth segment is the dominant cell and is the pacemaker in this heart.

We wish to thank Prof. Y. Nisimaru for his encouragement, and also it is our privilege to acknowledge our sincere gratitude to Prof. C. A. G. Wiersma, Prof. T. H. Bullock and Dr. L. A. Woodbury for their help in reviewing the manuscript. We are also grateful to the grant of the Ministry of Education of Japan for a part of our apparatus which made the work possible.

Summary

1. The electrocardiogram of the tubular heart of the mantis shrimp, *Squilla oratoria* de Haan, was studied. Structural findings are described which confirm Alexandrowicz's observation.

2. The electrogram consists of rapid spike components and slow action potentials. The spikes originate from the median nervous system of this heart, and the slow potential from the muscle.

3. The results of transection experiments on the nerve trunk support the view that the nerve cell of the thirteenth segment has a dominant role in the pacemaker activity of the heart contraction.

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THE AMINO ACID CONSTITUENTS OF THE PHYCOBILIN CHROMOPROTEINS OF THE RED ALGA PORPHYRA[1]

RAYMOND F. JONES[2] AND L. R. BLINKS

Hopkins Marine Station of Stanford University, Pacific Grove, Calif.

The phycobilin chromoproteins, phycocyanin and phycoerythrin of red algae, because of their stability and water-solubility, have been studied physico-chemically by many investigators. They have been shown to have definite molecular weight, characteristic isoelectric points, mobility, diffusion and adsorption properties (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Svedberg and Eriksson, 1932; Tiselius, 1930, 1937; Swingle and Tiselius, 1951). Their behavior as accessory pigments in photosynthesis is also well established (Haxo and Blinks, 1950; French and Young, 1952; Blinks, 1954a, 1954b; Yocum and Blinks, 1954). A comparative study of the chromatographically separated phycobilins of red and blue-green algae has recently revealed the occurrence of individual phycobilin pigments other than the classical varieties (Haxo, O'hEocha and Norris, 1955; Tiselius *et al.*, 1956). The former authors also established the presence of allophycocyanin as a natural, (although minor) component of the chromoproteins of several of the red and blue-green algae. The water-soluble chromoproteins of *Porphyra naiadum*, after separation by column chromatography and electrophoresis, have been shown to contain an appreciable quantity of allophycocyanin as well as phycoerythrin and phycocyanin (Airth, 1955; Blinks and Airth, 1957). A highly ionized lavender fraction was also found to be present. This behaved as a homogeneous entity and moved as an anion in the electrophoretic cell even at a pH of 5.0 where phycocyanin and phycoerythrin are nearly isoelectric.

Quantitative analyses of crystallized chromoproteins by Kylin (1910), Kitasato (1925) and Fujiwara (1955) show very little difference in elementary composition. Wassink and Ragetli (1952) reported on the amino acid composition of phycocyanin isolated from a species of the blue-green alga *Oscillatoria*. These authors detected sixteen ninhydrin-reactive spots of which thirteen were identified.

The present paper is concerned with the amino acid composition of several chromatographically pure phycobilin chromoproteins isolated from *Porphyra naiadum*, *Porphyra perforata* and *Porphyra Nereocystis*, all primitive red algae.

Material and Method

The algae were freshly collected from the shores of the Monterey Peninsula, California. *P. Nereocystis* was found growing epiphytically upon the stripes of the large kelp *Nereocystis Luetkeana*. *P. naiadum* was collected from the leaves of the

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[2] Present address: Atomic Energy Authority of U. K., Windscale Works, Cumberland, England.

flowering plant, *Phyllospadix* growing at mean low tide. The species *P. perforata* was collected from the rocks high up in the intertidal zone. Each species occupied, therefore, a different ecological habitat.

Extraction of pigments

The algae were returned to the laboratory in sea water, then washed twice with distilled water before extraction. In the case of *P. naiadum* the washed tissue was covered with distilled water and allowed to stand in the refrigerator for 24 hours at 5° C. (see Blinks and Airth, 1957). With *P. Nereocystis* and *P. perforata* it was found necessary to macerate the algal tissue in a Waring Blendor for two minutes and to allow the macerated material to stand in the refrigerator for 48 hours at 5° C. to obtain maximum extraction. The material was then filtered through cheesecloth and the filtrate passed through Whatman No. 1 filter paper pulp to remove chloroplasts and other fine organic debris. In each case the clear filtrate, exhibiting intense fluorescence, was precipitated with ammonium sulphate. Although the extracted chromoproteins of *P. Nereocystis* and *P. perforata* were precipitated with ammonium sulphate at 50% saturation, with *P. naiadum* precipitation was only complete at 90% saturation. After the purple-red precipitate was allowed to settle out overnight in the refrigerator it was removed by centrifugation, redissolved in distilled water and dialyzed against running tap water for 24 hours at 10° C., followed by 0.1 *M* acetate buffer at pH 5.0 for a further 24 hours. The non-dialyzable pigment solution was finally concentrated by pervaporation.

Fractionation

For the separation of the individual phycobilins the concentrated solution was subjected to column chromatography as employed by Airth (1955). Columns were prepared using one part tricalcium phosphate (dry weight) to five parts of washed Celite filter air. Gentle suction was used in forming the column which was washed with NaCl (1%) followed by 0.1 *M* acetate buffer at pH 5.0. The pigment extract was introduced onto the column and the individual phycobilins eluted with the appropriate buffer solutions, details of which are shown in Table I. Each fraction was further chromatographed on individual columns to ensure complete separation and elution.

The phycobilins from *P. naiadum* were also separated using a Tiselius electrophoresis apparatus. At pH 5.0 with acetate buffer, the individual chromoproteins were found to be homogeneous.

Absorption spectra were determined for each pigment over the range 250–700 mμ using a Beckman Model DU spectrophotometer.

The fractions, phycocyanin, phycoerythrin, allophycocyanin and the "highly ionized fraction" from *P. naiadum* were concentrated by pervaporation and a quantitative analysis of the amino acids released on acid hydrolysis undertaken. Because of the small amount of *P. Nereocystis* available at the time of the experiments, only phycocyanin and phycoerythrin were investigated from this alga.

Acid hydrolysis

The protein fractions were hydrolyzed using a mixture of equal volumes of concentrated hydrochloric acid and glacial acetic acid containing 4 per cent of stannous

TABLE I

Buffer solutions used in the chromatographic separation of the various phycobilins

Species	Phycobilin	Buffer	Absorption data
P. naiadum	Phycocyanin	1 *M* acetate pH 5	λ max. 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 545 mμ "Shoulder" 560–565 mμ
	Highly ionized fraction	2 *M* acetate pH 5	λ max. 565 and 615 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ
P. perforata	Phycocyanin	1 *M* acetate pH 5	λ max. 557 and 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 495 and 565 mμ "Shoulder" 540–550 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ
P. Nereocystis	Phycocyanin	1 *M* acetate pH 5	λ max. 557 and 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 495 and 565 mμ "Shoulder" 540–550 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ

chloride dihydrate (Fowden, 1954). Protein concentrations were adjusted to about 10 mg. per ml. and the hydrolysis performed in sealed tubes heated at 105° C. for 24 hours. After completion of hydrolysis the mixtures were evaporated to dryness *in vacuo* to remove the volatile acids, and the residues redissolved in 5 ml. distilled water. The amino acids were absorbed on the cation exchange resin Zeo-Karb 225, eluted with 1 N NH_4OH and finally dried in a vacuum desiccator over $CaCl_2$ and NaOH. Before chromatographing, the amino acids were taken up in 0.2 ml. isopropanol (10%) to which was added a little HCl.

Chromatographic procedure

Whatman No. 1 chromatography paper was used throughout. Spots were applied to the paper with calibrated micropipettes, the size of the spot being kept as small as possible (about 5 to 8 mm. diameter).

For two-dimensional chromatography, n-butanol: acetic acid: water (4:1:5) was used for the first direction and phenol-water (80:20) containing 0.04% 8-hydroxyquinoline in an atmosphere of NH_3 (1%) for the second direction. The butanol solvent was run for 28 hours at 20° C. and the solvent allowed to drip on the paper. The phenol solvent was run for 24 hours at a temperature of 20° C. The butanol was removed from the paper by drying the sheets in a current of warm air for two hours. The phenol was removed by the ether-wash technique of Fowden (1951). The amino acids phenylalanine, leucine and isoleucine were resolved

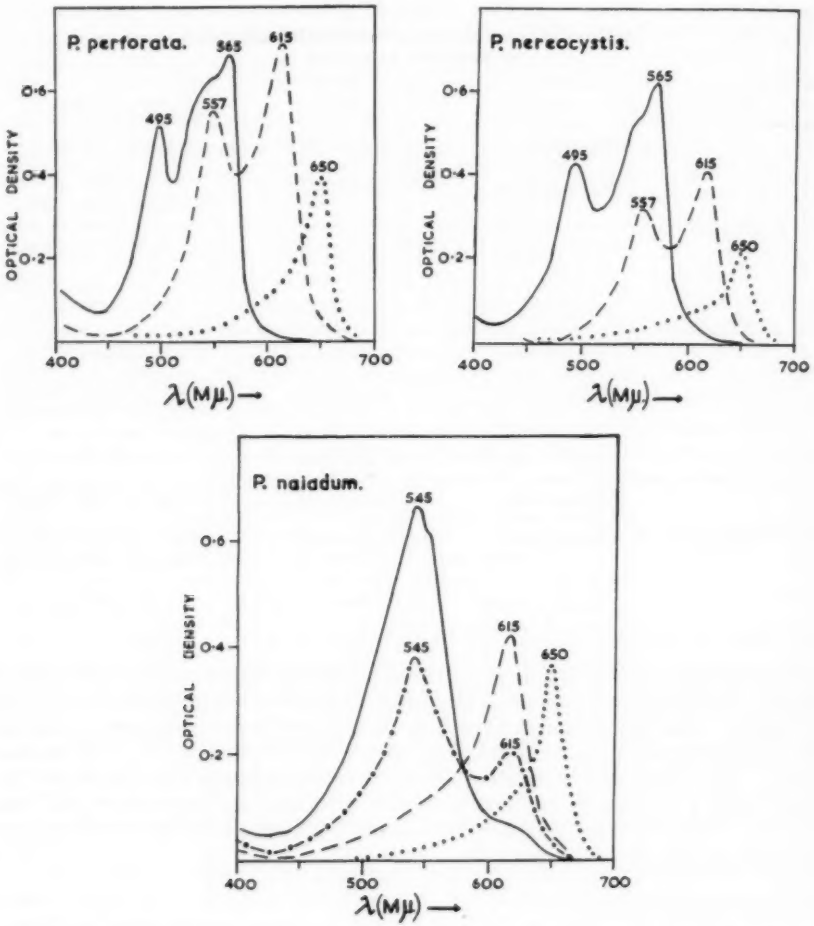

FIGURE 1. Absorption spectra of isolated phycobilins from three species of *Porphyra*. ———— phycoerythrin; – – – – – – phycocyanin; . . . . . allophycocyanin; –·–·–·– highly ionized fraction.

by one-dimensional chromatography using continued development for four days in water-saturated tertiary amyl alcohol in an atmosphere of 1% diethylamine. The solvent was removed from the paper by drying in a current of warm air for two hours.

The amino acids were located by dipping the chromatograms in 0.2% isatin in acetone and heating at 100° C. for three minutes to reveal proline and then dipping them into 0.2% ninhydrin in acetone and heating at 100° C. for 15 minutes to detect the other amino acids (Jepson and Smith, 1953; Smith, 1953).

TABLE II

Amino acid composition of phycobilin chromoproteins isolated from three species of Porphyra (Percentage by weight)

Amino acid	*P. naiadum*				*P. perforata*			*P. Nereocystis*	
	PC	PE	APC	"I"	PC	PE	APC	PC	PE
Aspartic	12.9	15.3	12.8	12.9	10.7	12.5	9.4	12.6	11.3
Glutamic	12.3	13.9	16.3	17.5	12.9	10.1	11.2	12.5	8.7
Serine	3.1	0.2	3.5	3.1	2.4	2.5	3.5	8.9	8.0
Glycine	5.4	5.7	7.2	5.2	6.8	9.1	9.2	11.9	13.5
Threonine	5.8	1.6	8.6	3.7	6.1	3.6	4.5	5.7	2.8
Alanine	12.3	18.3	10.9	9.5	12.6	20.3	12.5	12.3	11.7
Histidine	1.0	2.1	+	0.8	1.4	2.1	1.0	1.3	1.9
Lysine	2.6	1.4	5.1	2.3	3.1	4.9	3.6	3.4	6.5
Arginine	2.0	1.1	5.8	1.4	2.0	4.6	4.2	2.9	7.1
Proline	5.8	4.1	6.2	4.9	5.1	2.9	5.2	3.8	4.6
Valine+methionine	10.6	7.4	11.7	6.0	10.5	9.1	6.9	6.3	3.1
Phenylalanine	6.5	10.7	3.1	6.3	2.0	4.2	7.4	3.4	5.6
Leucine	11.2	10.1	5.1	10.5	12.2	8.9	11.1	7.8	6.8
Isoleucine	9.1	6.3	3.9	8.8	6.8	4.4	7.8	3.1	5.2
Tyrosine	−	2.0	+	5.0	4.4	1.9	+	4.1	3.7
Cystine	−	+	−	+	−	+	−	−	+

PC = phycocyanin; PE = phycoerythrin; APC = allophycocyanin; "I" = highly ionized fraction. + = present but too small to determine. − = not detected.

Quantitative estimation

The chromatograms, developed as described above, were utilized for quantitative estimation by a densitometric method. The maximum spot color density (*i.e.*, average blank reading minus the minimum reading for the given spot) multiplied by the spot area is a constant under the same conditions (Block, 1950). A densitometer suitable for the analysis of the chromatograms was constructed; this consisted of a photoelectric cell, a constant voltage light source and a galvanometer (Weston Model 440 No. 10623). Rapidity of operation was improved by fixing the photoelectric cell on a movable arm which held it over a circular light source of diameter 0.5 cm. Before use the light source was adjusted to produce a suitable standard transmission. For the paper blanks percentage transmission readings of 90–100 were obtained while the amino acid spots varied between 5 and 80 per cent transmission for the concentrations employed. The area of the amino acid spot was determined by tracing the spot on uniform paper and weighing the cut-out spot with a torsion balance. Standard chromatograms of known amino acid composition were developed at the same time as the hydrolysates. For the standard amino acids linear relationships were obtained over the range of 1–25 μgm. amino acid with an error of ± 12%.

All analyses were carried out in duplicate for each alga.

RESULTS

The three species of *Porphyra* investigated exhibit a graded increase in the ratio of phycocyanin to phycoerythrin. The deep water form, *P. Nereocystis*, has the

least phycocyanin. *P. naiadum* possesses comparatively large amounts of both phycocyanin and phycoerythrin. *P. perforata* has proportionately the most phycocyanin and the least phycoerythrin. Allophycocyanin is present in all three species of *Porphyra,* but is most abundant in *P. naiadum.* The absorption spectra for the various isolated and chromatographically purified phycobilins are shown in Figure 1.

The amino acid composition of these chromoproteins is given in Table II. The distribution of the amino acids is expressed as a percentage of the total weight of the amino acids in the protein. It is seen that, in the phycobilins isolated, the same

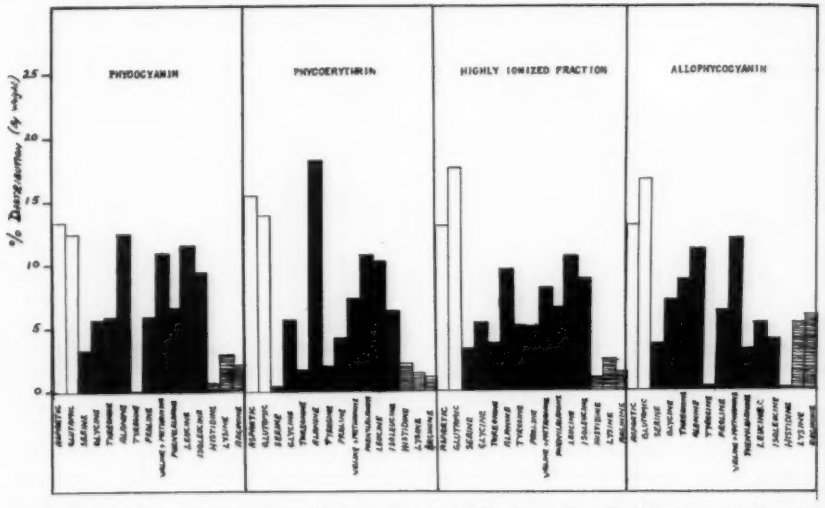

FIGURE 2. The major amino acid components of four chromoprotein fractions of *Porphyra naiadum.* Acidic amino acids are shown in white, basic, cross-hatched, and neutral, in black. The amounts are indicated as percentage of the total.

amino acids are present. Although tyrosine was not detected in the phycocyanin from *P. naiadum* it may well be due to the fact that this amino acid exists in concentrations considerably lower than that of any other amino acid present. The quantitative distribution of each amino acid varies for each protein analyzed. In all cases the dicarboxylic amino acids predominate and are comparable. Of the neutral amino acids, alanine and the two leucines are highest, although in the phycoerythrin isolated from *P. Nereocystis* glycine is present in relatively high concentration. It is interesting to note that alanine is present in greater amount than any other amino acid in the phycoerythrins isolated from *P. naiadum* and *P. perforata.* The basic amino acids of all the phycocyanins are low compared with the other phycobilin proteins. A histogram of the distribution in *P. naiadum* is shown in Figure 2.

DISCUSSION

The absorption characteristics of the isolated chromoproteins described in this work agree closely with those published by previous workers (Blinks, 1954a, 1954b; Airth, 1955; Haxo, O'hEocha and Norris, 1955). Typical R-phycoerythrins of the

higher red algae (Florideae) display absorption peaks at 495, 545, and 565 mμ. Of the lower red algae (Bangiales) here investigated, *P. perforata* and *P. Nereocystis* lack a pronounced second peak at 545 mμ. In *P. naiadum*, the phycoerythrin is characterized by a single peak at 545 mμ and a small shoulder at 560 mμ. This phycobilin has been designated B-phycoerythrin (Blinks, 1954b; Airth and Blinks, 1956), and is closest to the C-phycoerythrin of the Cyanophyta which has a single peak at 550 mμ. The phycocyanins of *P. perforata* and *P. Nereocystis* are similar in possessing the characteristic principal maximum at 615 mμ and a small one at 557 mμ. *P. naiadum*, however, contains a phycocyanin which has but a single absorption peak at 615 mμ. This is similar to the C-phycocyanin of blue-green algae. Allophycocyanin with an absorption maximum at 650 mμ is present in all three species of *Porphyra*, thereby substantiating the findings of Haxo, O'hEocha and Norris (1955) that it is a natural, although minor, component of the chromoproteins of marine algae. The highly ionized chromoprotein found only in *P. naiadum* has two absorption maxima, a major one at 545 mμ and a minor one at 615 mμ (probably due to the presence of phycocyanin).

The comparison of the above phycobilin proteins was undertaken to establish whether similar types of protein were present in the different species. The basis of comparison employed here, namely amino acid composition, although useful is subject to certain limitations. The physico-chemical properties of proteins depend not only upon their amino acid composition, but upon the arrangement of the amino acid residues within the protein and the nature of the helical configuration of the molecule. It is therefore realized that an amino acid analysis alone cannot account for all the biological or physico-chemical characters of the proteins.

The data presented show that the amino acid compositions of the various phycobilins differ significantly. Although *P. perforata* and *P. Nereocystis* possess phycobilins of similar absorption spectra, the amino acid composition of the proteins varies. The amino acid analysis of the phycocyanin from *P. naiadum* differs widely from the analysis published by Wassink and Regetli (1952) for the C-phycocyanin of *Oscillatoria* which has similar absorption characteristics. Of particular note is the presence of arginine which was absent from *Oscillatoria* phycocyanin. *P. naiadum* possesses phycobilin chromoproteins which differ from the other species of *Porphyra*, both in amino acid composition and absorption spectra. This is of particular interest since Professor G. J. Hollenberg (University of Redlands) has noted several morphological peculiarities which will probably remove *P. naiadum* from its present genus. The amino acid analysis of the highly ionized fraction present in this species offers little explanation for the high mobility of the molecule when subject to electrophoresis at pH 5.0. The degree of ionization is too great to be accounted for by the carboxyl groups of the amino acids. However, this fraction exhibited high absorption in the U. V. range of 265–280 mμ which suggests that the pigment may be attached to a nucleoprotein, in which case nucleic acid could be responsible for the high mobility.

Summary

1. The phycobilin chromoproteins of three species of *Porphyra* have been separated by column chromatography and their individual absorption spectra recorded. These "chromatographically purified" chromoproteins were subjected to acid hydrolysis and their constituent amino acids resolved by paper chromatography and determined quantitatively by a densitometric method.

2. The quantitative amino acid composition of each chromoprotein differed. The dicarboxylic amino acids alanine, glycine and the two leucines were most abundant. Alanine was found to be present in high concentration in the phycoerythrin of *P. naiadum* and *P. perforata*.

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HISTOLOGICAL CHANGES IN REGENERATING PIECES OF DUGESIA DOROTOCEPHALA TREATED WITH COLCHICINE

MARY A. McWHINNIE AND MARY M. GLEASON

Department of Biological Sciences, De Paul University, Chicago, Illinois

It has been shown that colchicine inhibits or completely abolishes regenerative changes in pieces of *Dugesia dorotocephala* (McWhinnie, 1955). Because of its selective stathmokinetic action it can be assumed that cellular studies, of colchicine-treated planarian pieces, should give evidence as to the source of blastema and regenerate cells. As early as 1902 Stevens reported numerous parenchymal cells in stages of mitosis, when untreated short transverse pieces of *Planaria lugubris* were regenerating. He suggested the embryonic nature of these cells which through multiplication and differentiation replaced the elements lost in section. With the use of x-rays Bardeen and Baetjer (1904) showed marked inhibition of regeneration in *P. maculata* and *P. lugubris*. Histological study showed no change in the cells of muscle, nerve, endoderm or gonad and an absence of mitosis in parenchymal cells. Control pieces showed mitotic cells in the parenchyma. Wiegand (1930) also demonstrated this point with several planarian species. On an indirect basis Curtis and Schultze (1934) emphasized the role of parenchymal cells in regeneration by comparing their number in species known to have high regenerative capacities (*P. maculata; P. agilis*) with one limited in regenerative ability (*P. fluviatilis*). Subsequent studies (Curtis, 1936) with x-rays showed a reduction of free parenchymal cells in proportion to the reduction in regeneration.

Colchicine inhibition of development has been shown by Beams and Evans (1940). Fertilized eggs of *Arbacia punctulata* were unable to divide if exposed to colchicine during the pre-metaphase interval and also showed a considerable decrease in viscosity.

Despite the evidence for the role of parenchymal cells in planarian regeneration, several workers have reported the absence of mitosis in normal planarian regeneration (Steinmann, 1926; Bandier, 1936; Clement, 1944). In an effort to demonstrate a specific source of cells which contribute to planarian regeneration, histological studies were made at selected intervals after colchicine treatment.

Materials and Methods

A stock of *Dugesia dorotocephala* was collected and maintained in the manner previously described (McWhinnie, 1955). Animals were sectioned into two halves at the level of the mouth. After sectioning, the pieces were separated into two groups. In one group, anterior and posterior halves were placed into $M/5000$ colchicine at the time of section. These were prepared for study at the end of exposure periods 3, 6 and 10 days. Pieces in the second group were placed into aerated tap water and were allowed to reconstitute for 24, 48 and 72 hours. At the end of each time interval these pieces were transferred to $M/5000$ colchicine where they were

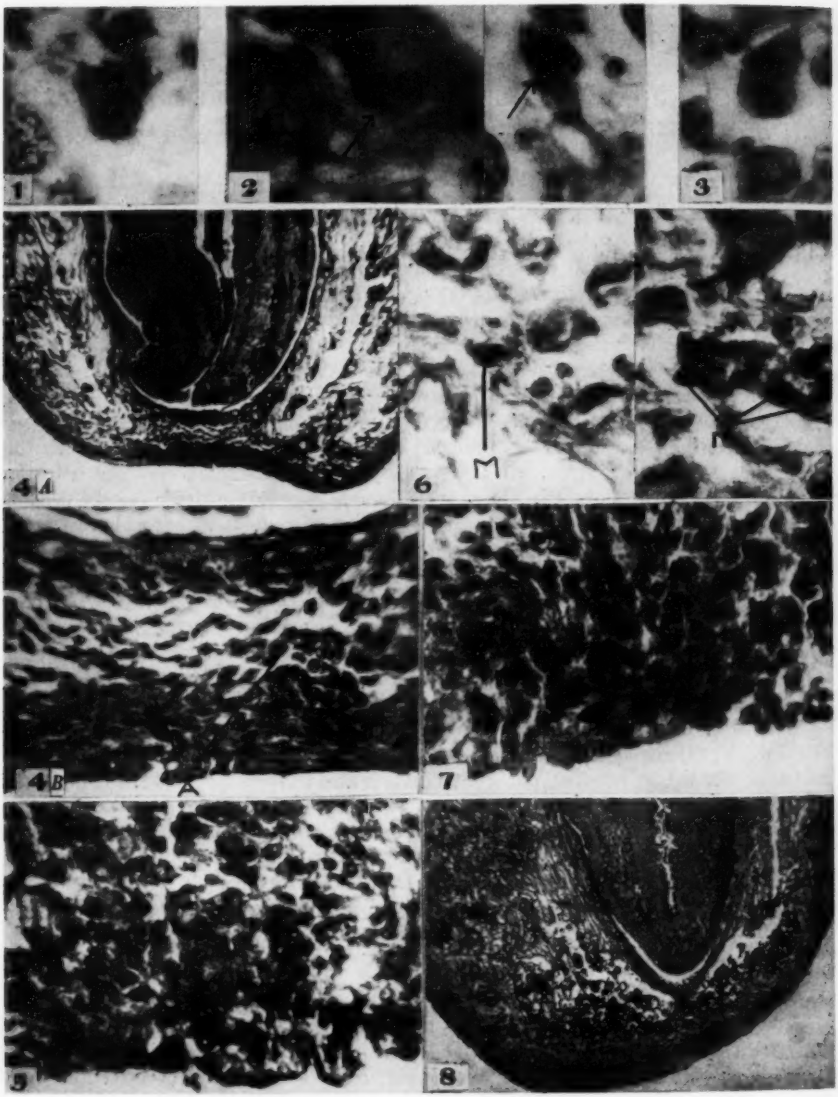

PLATE I

Explanation of Figures

FIGURE 1. Mitotic figure in a free amoebocyte in a three-day regenerating planarian piece, exposed to $M/5000$ colchicine from the time of section. × 1425.

permitted to remain for 12 and 24 hours. All pieces to be prepared for cell study were narcotized in 0.05–0.1% chloretone, killed and fixed in Bouin's fluid and embedded using the standard paraffin technique. These were sectioned at 6 micra in such a manner that both frontal and sagittal sections were obtained. Delafield's hematoxylin was used without a counterstain. Study was with 1000 and 1500 diameters magnification.

Results

The history of parenchymal cells in regenerating planarian pieces treated with colchicine demonstrates the paramount role of these cells in this developmental process. Planarian pieces treated with $M/5000$ colchicine for three days following section show many free amoebocytes in mitosis. These are uniformly distributed with extremely few in the area of the cut surface. Many of these cells appear elongate and in strands oriented to the cut surface, indicating migration. Mitotic figures were normal (Fig. 1). However, after 6 days' exposure to colchicine from the time of section most of the mitotic amoebocytes had abnormal chromosomal configurations and considerable pycnosis (Fig. 2). At 6 days these cells were still generally distributed throughout the parenchyma with never more than one to two in the region of the cut surface. Also, the large free amoebocytes were oriented into longitudinal strands. At the end of 10 days' exposure to colchicine a larger number of cells were at metaphase or beyond. However, at this time there was extensive cellular degeneration as evidenced by granulation of the nucleus, mitotic abberations and cytoplasmic vacuolation. Of the free amoebocytes undergoing degeneration many were oriented into migration strands.

Despite the apparent increase in number of parenchymal cells in mitosis from three to ten days after section in treated regenerating planarian pieces, the untreated pieces do not show this same progressive increase in number of cells undergoing division in time. Three days after section, untreated planarian pieces show some free amoebocytes in mitosis (Fig. 3) but none were observed in the region of section. On a comparative basis the number of mitotic figures was less than in the three-day colchicinized pieces. A marked decrease in the proliferation of these cells is apparent by six days after section when pieces regenerate in aerated tap water. However, at this time orientation of migratory strands to the area of the cut is

Figure 2. Mitotic figures in free amoebocytes in a six-day regenerating planarian piece, exposed to $M/5000$ colchicine from the time of section. × 1425.

Figure 3. Mitotic figure in a free amoebocyte in an untreated three-day regenerating planarian piece. × 1425.

Figure 4. A. Long section through the cut surface of a planarian piece treated with $M/5000$ colchicine for 24 hours after 24 hours in water. × 150. B. Same as A. × 660. (A = amoebocyte.)

Figure 5. Long section of an untreated regenerating planarian piece 48 hours after section. × 660.

Figure 6. Parenchyma of a planarian piece treated with colchicine for 24 hours after 48 hours in water. Note free amoebocytes in metaphase and evidence of migration. × 1425.

Figure 7. Long section of an untreated regenerating planarian piece 72 hours after section. Note increased number of amoebocytes. × 660.

Figure 8. Long section of a planarian piece treated with $M/5000$ colchicine for 24 hours after 72 hours in water. An increase in the number of cells can be seen in the region of the cut surface. An increase in gut degeneration is apparent. × 150.

notable. By ten days after section, when the regeneration process is conventionally considered complete, there is no evidence of mitotic activity. Regenerating pieces of planaria had no mitotic activity in epidermis, muscle or endoderm tissue, whether the pieces had been treated with colchicine or permitted to regenerate in water.

When anterior and posterior halves of planarians were maintained in water for 24 hours and then transferred to $M/5000$ colchicine for 12 and 24 hours, large numbers of free parenchymal amoebocytes were in metaphase with considerably fewer in telophase. These cells had divided *in situ* as well as during migration to the area of the cut surface. Degenerative changes in the gut and parenchyma were not found after 12 hours and were minimal after 24 hours treatment. In this group some few mitotic cells showed degenerative changes as indicated by granulation of nuclear components and rupturing of the cytoplasm. Cells of the gut, fixed nuclei of the syncytium, muscle and epidermis were not in mitosis. Beneath the newly-formed epidermal covering there was a slightly larger number of free amoebocytes than in the rest of the parenchyma (Fig. 4 A and B). At this same time interval untreated pieces had extremely few mitotic cells but a considerably greater number of amoebocytes at the cut surface (Fig. 5).

A similar group of anterior and posterior halves was allowed to undergo regeneration for 48 hours before treatment with colchicine for 12 and 24 hours. These pieces showed a still greater accumulation of free amoebocytes and more metaphase figures beneath the epidermal covering than in the previous group, *i.e.*, colchicine treatment after 24 hours in water. Many amoebocytes *in situ* and in migration were at metaphase (Fig. 6).

In untreated 72-hour regenerates there were few mitotic figures, but numerous amoebocytes were densely packed at the region of the blastema (Fig. 7). Pieces treated for 24 hours showed fewer mitotic figures than those treated for 12 hours. Treatment with colchicine for 12 and 24 hours, after a reconstitution period of 72 hours in water, resulted in pieces with a greater number of amoebocytes in the region of the cut surface. At this time fewer mitotic cells were seen in all areas. Gut, as well as general parenchymal degeneration was more apparent than in the previous series (Fig. 8). On the other hand controls showed large numbers of amoebocytes in the blastema, little evidence of mitotic activity and no degenerative changes.

Discussion

Through the use of colchicine on regenerating planarian pieces it can be concluded that the cellular elements involved in restoration to wholeness are primarily the free amoebocytes of the parenchyma. In all cases observed, after a minimum of 24 hours of reconstitution, epidermis, co-extensive with the epidermis of the rest of the piece, covered the wound surface. In no case was mitosis observed in this tissue. Similar wound epithelial covering without cell proliferation has been demonstrated in amphibian limb regeneration (Lash, 1955). The cut surface is readily distinguishable from the rest of the piece by the lack of sub-epidermal pigment as well as by the localized density of the parenchymal cells. With the use of colchicine it would appear that the time course of mitotic activity during regeneration explains the short interval in which there is no apparent change after section, the time of onset of greatest cellular proliferation and the known difference in susceptibility to toxic influences through the regeneration period. Some studies made in this work show

that mitotic cells increase in number from the third to the tenth day after section when pieces are placed into colchicine at the time of section. However, observation of untreated pieces at the same time intervals strongly indicates a greater number of parenchymal cells in division at the third day after isolation than in pieces 6 or 10 days after isolation. The progressively larger number of metaphase cells found at longer time intervals after isolation and introduction into colchicine would simply emphasize the sustained inhibition in the presence of the alkaloid and consequently the accumulation of inhibited cells.

Evidence can be gained as to the time of greatest mitotic activity by permitting isolated pieces of planarians to initiate normal regeneration in water before placing them into colchicine. Halves of planarians placed into colchicine for 24 hours after initial regeneration in water for 24, 48, and 72 hours show a greater number of parenchymal cells in division at a final age of 72 hours (48 hours in water, 24 hours in colchicine). However, a substantial number of these cells were in division in pieces placed into colchicine for 24 hours after 72 hours' regeneration in water. While the number of mitotic figures found in this group was less than in the preceding it is also true that parenchymal degeneration was considerably greater than in that group. Under these conditions there is extensive gut degeneration, granular degeneration in patches of parenchyma as well as in the area of the cut surface. Mitotic cells at this time showed a range from normal metaphase to cells with chromosomes widely dispersed and granular in appearance. These modifications were not so apparent in pieces placed into colchicine after 48 hours in water.

The fixed 24-hour exposure to colchicine in these three groups with a greater susceptibility of pieces at the fourth day of reconstitution agrees with previous findings on the critical period in reconstitutional development. By studies of gross changes in planarian pieces and their susceptibility it was demonstrated that the fourth day in development is the most critical (McWhinnie, 1955).

While the highest mitotic activity appears at the third day after isolation and both gross and microscopic evidence show a high susceptibility at the fourth day, it would appear that the increased population of parenchymal cells and their migration to the cut surface constitute the most active and therefore the most susceptible period in planarian regeneration. This is visibly expressed by the toxic effects of colchicine on the fourth day.

It is suggested that the sequence of events in planarian regeneration includes an initial slow onset of division of parenchymal cells, rising to a peak at the third day after isolation. Associated with the rise in number of cells proliferating, oriented migrations of these cells to the cut surface follows. It would appear that the activity of migration is greatest through the fourth to the sixth day afer section. Some oriented strands found in 48-hour pieces indicate the onset of migration at this time. By the sixth day of regeneration, mitotic activity and cell migrations are subsiding and the remainder of the reconstitution period represents the time of morphogenetic changes to complete species organization, both internal and external.

It can be concluded also that the mechanism of colchicine inhibition of planarian regeneration is through its influence on parenchymal amoebocyte proliferation as well as reduced migration of those cells to the cut surface(s). It is entirely likely that colchicine-induced changes in viscosity (Beams and Evans, 1940) could account, in part, for the marked difference in cell density in the blastema of normal and treated regenerating pieces.

SUMMARY

1. Histological studies show that the mechanism of colchicine inhibition of regeneration in pieces of *Dugesia dorotocephala* is the stathmokinetic action it exerts on free parenchymal amoebocytes.

2. Parenchymal amoebocytes are the only cells exhibiting mitotic activity during the period of regeneration.

3. Mitotic activity reaches a peak at the third day of development while oriented migrations of amoebocytes appear to set in at the second day with marked movement through the fourth to the sixth day after section.

4. The free amoebocytes of the parenchyma constitute the exclusive source of cells participating in the replacement of parts lost when planarian worms are sectioned.

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GROWTH-PROMOTING EFFECTS OF HYDROLYZED NUCLEIC ACIDS, NUCLEOTIDES, AND NUCLEOSIDES ON ENDAMOEBA HISTOLYTICA

MITSURU NAKAMURA

Department of Bacteriology, Montana State University, Missoula, Montana

Endamoeba histolytica has not yet been maintained indefinitely in pure culture, although bacteria-free cultures have been carried for periods of up to one month in growth factor-fortified media (Nakamura and Baker, 1956). Jacobs (1947) and Shaffer and Frye (1948) have also grown the amebas in media containing no or relatively few multiplying bacteria. Therefore, it becomes apparent that although bacteria contribute tremendously to the growth and multiplication of the amebas, they are not absolutely essential and that perhaps amebic growth can occur in a semi-synthetic medium if supplied the necessary growth-promoting factors. It has been shown that purines, pyrimidines, citrovorum factor, and ribose-5-phosphate can substitute partially for bacterial association and permit bacteria-free cultures of *E. histolytica* to multiply for a limited period. However, ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) tested singly or in combination failed to promote amebic growth; on the other hand, dialysates of media containing RNA and DNA preconditioned by bacterial growth contained ameba-stimulatory substances which permitted seven sub-cultures of the amebas in the absence of associated bacteria (Nakamura and Baker, 1956). It was postulated that bacterial action on the nucleic acids produced catabolic intermediate(s) which were essential to the nutrition of the amebas. In order to determine more exactly the specific components in the nucleic acid digest which were ameba-stimulatory, nucleic acids were hydrolyzed by enzymatic, acid, and alkaline hydrolysis; the dialysates of the hydrolysates were studied for their effects on *E. histolytica* under bacteria-free conditions. Furthermore, nucleosides and nucleotides, obtained from commercial sources, were also assayed for their activity on the growth of the amebas.

Materials and Methods

Organism and the assay medium

Strains of *E. histolytica* employed in these experiments consisted of: (1) NRS, obtained from Dr. Quentin M. Geiman, Stanford University School of Medicine, San Francisco, California, (2) HUS-100, isolated from the stool of a carrier during an outbreak of amebiasis in Indiana in 1953, obtained from Dr. Chia-Tung Pan, Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts, and (3) UC, also obtained from Dr. Pan. Stock cultures of the amebas, containing a mixed bacterial flora, were maintained in a modified Boeck-Drbohlav (1925) medium. The assay methods were essentially identical with those described earlier (Nakamura, 1955; Nakamura and Baker, 1956; Nakamura and

Jonsson, 1956). Coagulated egg slants were overlaid with a liquid phase consisting of glucose (0.5%), sodium thioglycollate (0.3%), penicillin G (10,000 units/ ml. final concentration), streptomycin (5000 units/ ml. final concentration), horse serum-Ringer solution (1/5), rice powder (approximately 10 mg.), and a vaspar (vaseline and paraffin, 1/1) seal. The volume of the overlay fluid was four ml. The dialysates and the nucleosides and nucleotides assayed were added to the liquid phase of the medium.

The inocula consisted of 2 drops of stock cultures adjusted to contain approximately 15–20 amebas per low power field. The bacteria introduced with the ameba inocula were sterilized within 4–6 hours by the combinations of antibiotics used. The culture tubes were incubated for 3–4 days at 37° C.; ameba counts were made by taking the sediment from each culture tube (in duplicate), placing a few drops on a clean slide, covering with a cover slide (22 × 22 mm.) and counting the number of amebas per low power field. Ten fields were counted and an average count recorded. At the same time the amebas were transferred to tubes containing identical nutritional components. Control tubes consisted of media lacking only the materials being assayed. Positive controls consisted of media fortified with the growth factors ribose-5-phosphate and adenosinetriphosphate.

Hydrolysis of nucleic acids

The method of Kerr *et al.* (1949) was used for the acid hydrolysis of RNA. Fifty mg. of RNA were placed in a test tube with 5 ml. of 2 N sulfuric acid. The tube was placed in boiling water for 30 minutes. After hydrolysis the contents of the tube were diluted to 25 ml. with water. Acid hydrolysis of DNA was accomplished by placing 50 mg. of DNA in 5 ml. N sulfuric acid and refluxing in boiling water for 2 hours. The hydrolysate was adjusted to pH 6.5 with alkali.

The method of Volkin and Carter (1951) was used for the alkaline hydrolysis of RNA. Fifty mg. of RNA, dissolved in 3 ml. of 0.5 N NaOH, were kept at 37° C. for 17 hours. The digest was diluted with water to 0.02 N NaOH and finally neutralized. DNA was hydrolyzed using a modified method of Marrian *et al.* (1951).

Enzymatic hydrolysis of RNA was accomplished by suspending 50 mg. of RNA in 5 ml. water and adjusting to pH 7.2 with dilute NaOH. Then 5 mg. of crystalline ribonuclease, dissolved in 1 ml. of 0.1 *M* phosphate buffer at pH 7.2, was added to the nucleic acid solution under stirring. As the reaction progressed the solution was maintained at pH 7.2 with the addition of 0.05 N NaOH. The temperature of the digest was maintained between 25–27° C. Hydrolysis was complete in two hours. The method of Smith and Markham (1952) was used for the enzymatic digestion of DNA; the DNA was digested with desoxyribonuclease (20 ugm./ml.) in 0.005 *M* magnesium sulfate at pH 7.0 for 18 hours. The digests were dialyzed in water and the dialysates tested for their growth-promoting activity.

Results

As is evident in Tables I, II, and III, enzyme-hydrolyzed nucleic acids (both RNA and DNA) yielded a product which was stimulatory to the growth of *E. histolytica*. In all of the experiments enzyme-hydrolyzed RNA consistently stimulated the amebas slightly more than the enzyme-hydrolyzed DNA preparation. Al-

TABLE I

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; strain NRS

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	15	1
Basal + enzyme-hydrolyzed RNA	4	79
Basal + enzyme-hydrolyzed DNA	4	55
Basal + alkaline-hydrolyzed RNA	4	47
Basal + alkaline-hydrolyzed DNA	4	50
Basal + acid-hydrolyzed RNA	4	0
Basal + acid-hydrolyzed DNA	4	0
Basal + unhydrolyzed RNA	8	10
Basal + unhydrolyzed DNA	8	7
Basal + adenosine (0.1 mg./ml.)	4	41
Basal + guanosine (0.1 mg./ml.)	4	49
Basal + thymidine (0.1 mg./ml.)	4	59
Basal + adenylic acid (0.1 mg./ml.)	4	70
Basal + guanylic acid (0.1 mg./ml.)	4	66
Basal + thymidylic acid (0.1 mg./ml.)	4	83
Basal + uridylic acid (0.1 mg./ml.)	4	47

TABLE II

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; strain HUS-100

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	15	0.4
Basal + enzyme-hydrolyzed RNA	4	64
Basal + enzyme-hydrolyzed DNA	4	40
Basal + alkaline-hydrolyzed RNA	4	51
Basal + alkaline-hydrolyzed DNA	4	54
Basal + acid-hydrolyzed DNA	4	1
Basal + acid-hydrolyzed RNA	4	0
Basal + unhydrolyzed RNA	4	0
Basal + unhydrolyzed DNA	4	1
Basal + adenosine (0.1 mg./ml.)	4	39
Basal + guanosine (0.1 mg./ml.)	4	34
Basal + thymidine (0.1 mg./ml.)	4	43
Basal + adenylic acid (0.1 mg./ml.)	4	57
Basal + guanylic acid (0.1 mg./ml.)	4	68
Basal + thymidylic acid (0.1 mg./ml.)	4	49
Basal + uridylic acid (0.1 mg./ml.)	4	33

kaline hydrolysates of nucleic acids were also stimulatory to the amebas. However, acid-hydrolyzed nucleic acids were without ameba-stimulatory properties in studies on all three strains of *E. histolytica*. Unhydrolyzed nucleic acids were inactive, except for a slight effect on the NRS strain, as was to be expected according to the earlier data of Nakamura and Baker (1956). The nucleosides adenosine, guanosine, and thymidine stimulated the HUS-100 and NRS strains but not the UC strain. The nucleotides adenylic acid, guanylic acid, thymidylic acid, and uridylic acid were active as growth factors for all three strains of amebas tested, although

TABLE III

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; UC strain

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	18	2
Basal + enzyme-hydrolyzed RNA	4	83
Basal + enzyme-hydrolyzed DNA	4	69
Basal + alkaline-hydrolyzed RNA	4	40
Basal + alkaline-hydrolyzed DNA	4	40
Basal + acid-hydrolyzed RNA	4	3
Basal + acid-hydrolyzed DNA	4	2
Basal + unhydrolyzed RNA	4	1
Basal + unhydrolyzed DNA	4	0
Basal + adenosine (0.1 mg./ml.)	4	0
Basal + guanosine (0.1 mg./ml.)	4	2
Basal + thymidine (0.1 mg./ml.)	4	5
Basal + adenylic acid (0.1 mg./ml.)	4	61
Basal + guanylic acid (0.1 mg./ml.)	4	68
Basal + thymidylic acid (0.1 mg./ml.)	4	90
Basal + uridylic acid (0.1 mg./ml.)	4	56

the degree of activity as growth-promoting factors varied slightly from compound to compound.

Attempts to maintain bacteria-free subcultures on media containing hydrolyzed nucleic acids, nucleotides, or nucleosides were generally unsuccessful. The longest culture maintained on enzyme-hydrolyzed RNA and enzyme-hydrolyzed DNA was 5 transfers for a total of approximately 15 days. Sterility tests indicated the absence of viable bacterial cells. In media containing alkaline-hydrolyzed nucleic acids, two to three subcultures were usually possible; however, the total amebic populations were considerably lower than in the enzyme-treated nucleic acid media. Only one subculture with a meager ameba count was possible in the experiments containing nucleosides and nucleotides as growth factors.

DISCUSSION

The data in this report are in agreement with earlier reports that nucleic acids preconditioned by bacterial growth produce some catabolic metabolite(s) which are essential for amebic growth in the absence of living bacteria. In these experiments, enzymatic and alkaline digestion, rather than bacterial preconditioning, yielded ameba-growth-promoting factors. It is indeed difficult to explain the absence of similar stimulatory activity in the acid-hydrolyzed nucleic acid solutions. In studies with *Trichomonas vaginalis*, Sprince *et al.* (1953) reported that acid hydrolysis of RNA destroyed the growth-promoting effect of RNA whereas alkaline hydrolysis of RNA left intact this growth-promoting factor. They also reported that acid, alkaline, and enzymatic hydrolysis of DNA destroyed the growth-promoting effects of DNA. These results, however, are not quite analogous to the data in this paper since Sprince *et al.* (1953) were dealing with DNA and RNA which were established as growth-promoting factors for *Trichomonas;* in the case of *Eudamoeba histolytica,* DNA and RNA in themselves do not stimulate amebic growth.

It is highly probable that the ameba-growth-stimulatory action of nucleic acid hydrolysates was not due solely to the nucleosides and nucleotides formed during the digestion. Smith and Markham (1952) have found that enzyme digestion of DNA produces many dinucleoside monophosphates; Markham and Smith (1952) reported that products of enzyme hydrolysis of RNA were largely cyclic pyrimidine nucleotides and only traces of adenylic and guanylic acids were found. On the other hand, alkaline digestion of RNA produces guanylic, adenylic, and uridylic acids (Magasanik and Chargaff, 1951).

The growth factor effects of purified nucleosides and nucleotides indicate the importance of these substances in amebic nutrition; these substances play a role in the synthesis of nucleic acids and pyridine nucleotides. Diphosphopyridine nucleotide has been shown to be necessary for amebic growth in the absence of bacteria (Nakamura and Baker, 1956). Johnson (1953) similarly showed that cytidylic and guanylic acids were growth factors for *Paramecium multimicronucleatum*.

There is evidence that different strains of *E. histolytica* possess different growth factor requirements. The nucleosides, which were highly active for the NRS and HUS-100 strains, were without activity on the UC strain. It is possible that the UC strain can synthesize its own nucleoside but that it cannot phosphorylate the nucleoside into the nucleotide which it apparently requires. In the cases of the NRS and HUS-100 strains, it appears logical to assume that they can synthesize neither nucleosides nor nucleotides, yet when supplied these two growth factors exogenously, the amebas can synthesize their own nucleic acids. A strong point in favor of this assumption is the fact that pre-formed nucleic acids do not aid amebic growth appreciably whereas the nucleosides and nucleotides are highly stimulatory.

Summary

1. Enzyme and alkaline hydrolysates of ribonucleic and desoxyribonucleic acids contained growth-promoting factors for *Endamoeba histolytica*. Acid hydrolysates of nucleic acids, however, were without this stimulatory activity on the amebas.

2. Nucleosides, adenosine, guanosine, and thymidine, were stimulatory to the NRS and HUS-100 strains but not for the UC strain. Nucleotides, adenylic acid, guanylic acid, thymidylic acid, and uridylic acid, were highly stimulatory for the growth of all three strains of *E. histolytica* studied.

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ANAEROBIC RECOVERY OF ASCARIS EGGS FROM X-IRRADIATION [1]

GEORGE PAHL [2] AND C. S. BACHOFER

Department of Biology, University of Notre Dame, Notre Dame, Indiana

Lea (1947) in his classic work on radiation biology has pointed out in his analysis of the work of Henshaw (1940) that the recovery of unfertilized, x-irradiated eggs of *Arbacia* is probably not due to diffusion out of the egg of inhibitory substances; the correlation between recovery rate and oxygen uptake suggests that the effect of the radiation is to destroy some nuclear constituent, and recovery consists in the re-formation of this constituent as a result of the metabolic activity of the cell. The rate of oxygen uptake is presumably an indication of the general level of metabolic activity, and in *Arbacia* eggs appears to vary in different stages of the egg in much the same way as does whatever reaction is responsible for recovery. It does not follow necessarily that the rates of recovery in different organisms will be proportional to their respective rates of oxygen uptake. Some organisms have, in fact, been shown to consume oxygen at appreciable rates after irradiation although they do not show any recovery.

The eggs of *Ascaris lumbricoides suum* possess certain advantages for a test of the question whether oxygen is necessary for recovery from x-irradiation. Since they are facultative anaerobes they can be held for long periods of time in anaerobic conditions. Even at optimal temperatures for normal development, under anaerobic conditions the eggs do not develop. If recovery should occur during the enforced anaerobic metabolism, not only would the necessity of oxygen uptake for recovery be disproved for *Ascaris* eggs, but some other possible mechanism of recovery would be suggested. The present paper complements a preliminary report (Pahl and Bachofer, 1954).

Materials and Methods

A stock of eggs of *Ascaris lumbricoides suum* in the one-cell stage was prepared according to methods already described by Bachofer and Pahl (1955). The source of x-rays was a beryllium-window tube operated at 100 kvp. and 8 ma., without added filtration. Each irradiated sample consisted of 10^5 eggs suspended in one ml. of distilled water and placed in an open, flat-bottom vial 2.7 cm. in diameter. The egg suspension was approximately 1.8 mm. deep with the eggs resting on the bottom during the exposure. The dose was calculated to be 12,000 r/min. at the center of the irradiated layer of eggs. This value was determined by exposing ferrous ammonium sulfate as a dosimeter to a 325-curie cobalt-60 source of gamma rays, as described by Weiss (1952). *Ascaris* eggs were then exposed to the gamma rays under the same conditions, and the biological response was correlated with dose.

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[2] Present address: St. Mary's College, Winona, Minnesota.

Aliquots of the same sample of eggs were then exposed to the x-ray beam, and from their response the output of the x-ray tube was determined. All subsequent exposures to x-rays were carried out under identical conditions at the same dose rate.

Each irradiated sample was diluted 20-fold with *Ascaris* physiological saline solution (Baldwin and Moyle, 1947) immediately after irradiation. Aliquots of two ml. each were de-oxygenated by bubbling specially purified nitrogen through the saline solution containing the eggs. The eggs were then placed at the appropriate incubation temperatures. After certain designated incubation periods, the seals were broken and the supernatant de-oxygenated fluid was drawn off while the eggs remained settled on the bottom. *Ascaris* saline, in equilibrium with air, was then added and incubation completed at 30° C. The same procedure was followed for non-irradiated controls.

TABLE I

Effect of post-irradiation anaerobic treatment at 15° C., 20° C., and 30° C. over a period of two weeks on the survival of x-irradiated Ascaris eggs. Dose: 28,000 r

Post-irradiation treatment		Per cent survival			
		Days under anaerobic conditions			
		0	1	7	14
30° C.	Aerated	47.5	45.0	47.3	48.8
	De-oxygenated	47.3	58.0	57.3	60.3
20° C.	Aerated	49.8	46.5	43.0	35.0
	De-oxygenated	45.0	48.3	50.0	38.8
15° C.	Aerated	48.8	42.0	36.0	27.8
	De-oxygenated	47.5	51.8	45.5	34.3

The two criteria used to determine the effects of irradiation and anaerobic treatment were the rate of first cleavage and the percentage of eggs which developed to the motile embryo stage. The term "survival" is used to designate the development of irradiated eggs into motile embryos.

RESULTS

Table I shows the results of irradiation of *Ascaris* eggs which were de-oxygenated immediately after irradiation and stored at temperatures of 15° C., 20° C., and 30° C. After periods of 1, 7, and 14 days at these temperatures, the de-oxygenated samples were aerated and incubated at 30° C. The results show that at any given temperature there was a higher survival for irradiated eggs given anaerobic treatment than for those incubated only aerobically. Keeping the eggs under anaerobic conditions for more than one day did not increase survival. Irradiated eggs kept at 30° C. throughout the entire post-irradiation period showed the highest survivals

TABLE II

Per cent survival of x-irradiated Ascaris eggs as affected by anaerobic treatment immediately after exposure

Dose in roentgens	Per cent survival				
	Days of post-irradiation anaerobic treatment				
	0	½	1	2	7
0	97.5	96.8	97.0	97.3	96.6
28,000	47.3	56.7	58.0	57.1	57.3
40,000	26.9	36.3	36.4	36.6	36.4
48,000	12.5	20.8	21.1	21.7	22.0
60,000	2.5	6.7	6.4	6.8	6.6

for both anaerobic and aerobic treatment. This confirms a previous study of the authors (Bachofer and Pahl, 1955) on post-irradiation temperature treatment of *Ascaris* eggs. In view of this, all other experiments were performed at 30° C., which is approximately the optimal incubation temperature.

Table II shows that anaerobiosis is able to bring about recovery over a wide dose range, even when untreated samples give survivals as low as 2.5%. When there was no anaerobiosis there was no recovery, recovery being represented by the difference in survival between the first column (where there was no anaerobiosis) and the other columns (where there was anaerobiosis). The critical period of recovery is shown to occur during the first 24 hours of anaerobiosis, since survivals for 12 hours of treatment are not increased appreciably for longer periods of treatment.

The effect of delaying the anaerobic treatment after irradiation was next investigated, with both cleavage delay and survival as criteria of recovery. The results summarized in Table III for cleavage delay show that the eggs must be de-oxygenated before 15 hours have elapsed after irradiation in order to procure recovery. The low values in the column for *no delay* in anaerobic treatment indicate the greatest recovery. Conversely, in Table IV, the high values in the column for *no delay* in anaerobic treatment indicate the greatest recovery. The crucial period of approximately 15 hours, therefore, affects both cleavage delay and survival.

TABLE III

50% cleavage time in hours for x-irradiated Ascaris eggs as affected by 24-hour anaerobic treatment initiated at various intervals after exposure

Dose in roentgens	No anaerobic treatment	Delay before anaerobic treatment		
		0 hrs.	15 hrs.	24 hrs.
	50% cleavage time in hours			
0	40.0	39.5	40.3	40.0
24,000	53.1	46.7	52.7	53.1
40,000	58.4	51.4	57.7	58.2
48,000	60.0	53.4	59.4	59.5

The recovery phenomena summarized above have been verified over a dose range of 24 to 48 kr, both for delay of cleavage and for survival.

Since anaerobic conditions during irradiation give high protection to *Ascaris* eggs, an experiment was designed to test whether post-irradiation anaerobic recovery could be secured with eggs which had been protected by anaerobic conditions during irradiation. When a dose of 60,000 r was delivered to one sample of eggs

TABLE IV

Per cent survival of x-irradiated Ascaris eggs as affected by a 24-hour anaerobic treatment begun at various intervals after exposure

Dose in roentgens	No anaerobic treatment	Delay of anaerobic treatment			
		0 hrs.	15 hrs.	24 hrs.	60 hrs.
	Per cent survival				
0	97.0	97.0	97.3	97.5	97.0
24,000	63.5	73.9	64.8	62.3	62.0
40,000	26.9	36.4	27.2	27.6	25.8
48,000	12.5	21.1	11.8	12.5	12.0

that was in equilibrium with air and to another similar sample that was under anaerobic conditions, the survival was increased in both cases by post-irradiation anaerobiosis. These increases in survival were duplicated when treatment for 1½ hours in 0.1 *M* KCN was substituted for post-irradiation anaerobiosis. Immediately after the period of exposure to cyanide, the eggs were washed by centrifugation and incubated in *Ascaris* saline. The results in Table V clearly indicate that the same pattern of protection can be obtained with cyanide as with anaerobiosis.

TABLE V

Effect of 24-hour post-irradiation treatments immediately following the irradiation of Ascaris eggs under different conditions during irradiation. Dose: 60,000 r

Treatment after irradiation	Treatment during irradiation	
	In equilibrium with air	Anaerobic
	Per cent survival	
Aerobic	2.5	74.3
Anaerobic	6.4	82.1
KCN	6.5	82.2

In other studies (unpublished results) the authors have established that cyanide inhibits the oxygen consumption of *Ascaris* eggs. Cyanide, however, is a general inhibitor of respiratory cycles whether they include the cytochrome system or not. To demonstrate that *Ascaris* eggs do have a cytochrome system, they were subjected to the light-reversal inhibition test of carbon monoxide by use of a Warburg respirometer adapted to this purpose. The eggs showed the same rate of oxygen consumption in air and in a 5% oxygen–95% nitrogen mixture. When CO replaced the nitrogen, however, there was an immediate and persistent drop in oxygen

consumption. Within a few minutes this leveled off at approximately 40% of normal consumption. In the presence of light this value rose to 75% of normal consumption.

Discussion

Numerous studies on the effect of anaerobiosis and other factors *during* irradiation are not comparable to the present investigation, since the present study utilizes anaerobiosis *after* irradiation and is therefore concerned with recovery processes. Although a number of post-irradiation treatments have delayed the expression of injury or decreased its rate of development, most of them have had no effect on the final outcome. In work with mice, Bacq *et al.* (1950) found that NaCN given immediately after irradiation only delayed mortality, and they concluded that cyanide was ineffective when given after irradiation. Bachofer (1956) has shown that post-irradiation anaerobiosis of x-irradiated *Ascaris* eggs restores in part the normal rate of pronuclear fusion, which is slowed down considerably by x-irradiation; the restoration is a genuine recovery and is attributable to the period of anaerobiosis.

The problem proposed by Lea (1947), as to whether the recovery of irradiated invertebrate eggs demands oxygen uptake or whether this uptake is a mere concomitant action, has been solved for *Ascaris* eggs. The facultative anaerobic nature of *Ascaris* eggs makes possible a complete elimination of free oxygen during incubation at optimum temperature. When x-irradiated eggs were subjected to post-irradiation anaerobiosis, their power of recovery surpassed that of x-irradiated aerated eggs as shown by decreased cleavage time and by higher survival (Tables I–V). It has been established, therefore, that oxygen is not necessary for recovery in this case.

A possible mechanism to be considered is whether this recovery could be attributed to the concomitant delay in cleavage brought about by anaerobic conditions. In studies concerned with cell cleavage, Schjeide and Allen (1951) found that tadpole hematopoietic cells appear to be susceptible to x-rays in direct proportion to the amount of cell division allowed to proceed following the irradiation period. Recovery of irradiated *Arbacia* eggs (Henshaw, 1940) was obtained only if they were kept unfertilized; as the time between irradiation and fertilization was shortened, the recovery was likewise decreased. In unirradiated *Arbacia* eggs cleavage begins at optimal temperatures within an hour after fertilization. Cytological observations (Bachofer, 1956) show that all eggs of *Ascaris lumbricoides* removed from the terminal 25 mm. of the uteri, in which the sperm has entered the egg, are in the pronuclear stage. Upon incubation at optimal temperature, pronuclear fusion begins slowly and precedes first cleavage by approximately 1½ hours. The eggs begin first cleavage only after 25 to 30 hours of incubation at optimal temperature, and achieve 50% cleavage after 40 hours. Since anaerobiosis had to be initiated before 15 hours had elapsed after irradiation in order to secure recovery, and anaerobic recovery was reduced if the treatment was delayed even a few hours following irradiation, it appears that the recovery process in question is not directly associated with the delay of first cleavage. Furthermore, if delaying the time of cleavage were the important factor in recovery, it would be expected that the survival of the irradiated eggs which were de-oxygenated and placed at the various sub-optimal temperatures would have remained at the same peak as those placed at 30° C. The progressive decrease in survival of both the aerated and anaerobically incubated eggs kept for

increasing lengths of time at temperatures lower than optimal (Table I) indicates that some factor other than cleavage delay is responsible.

Further evidence that cleavage delay is not the contributing condition for recovery is shown by the fact that the anaerobic incubation facilitates recovery only during the first 15 hours of this treatment (Table II). Likewise, eggs which have been allowed to incubate aerobically for 15 hours before being de-oxygenated give no evidence of recovery as indicated by cleavage delay (Table III) and survival (Table IV). During this 15 hours of aerobic incubation the eggs have not yet begun their first division. It appears that one must look to other conditions than delay of cleavage to explain the recovery.

It should be borne in mind that cellular activity, including cell cleavage, is necessary in most cases to demonstrate the injury, since the injury is latent. Postponement of cellular activity after irradiation may not involve recovery; once cellular activity is allowed to proceed the damage may be manifested. If the damage is as great as that which would have been manifested by permitting cellular activity to proceed immediately after irradiation, then there was no genuine recovery. True recovery was reported by Cook (1939) for survival of irradiated eggs of *Ascaris megalocephala* held at low temperatures after irradiation, but the opposite was found to be true for *Ascaris lumbricoides* (Bachofer and Pahl, 1955). Both studies agreed, however, in that post-irradiation treatment did not affect the time required for first cleavage. Pertinent to the present case, therefore, is the fact that forestalling cell cleavage and cellular activity after irradiation does not in itself produce genuine recovery.

The seat of the recovery from irradiation may involve various reactions of the respiratory cycle. The increased survival of irradiated eggs which have been subjected to cyanide or to anaerobiosis after irradiation suggests that the effects of irradiation operate to some extent through the cytochrome system, since both cyanide and anaerobiosis inhibit the cytochrome system. The mechanism of protection afforded by respiratory inhibitors may be either the prevention of the products of irradiation from reacting with the cytochromes or the prevention of the radiation-affected cytochromes from participating in the chain of reactions that normally bring about the observed effects of irradiation. Insofar as the cytochromes may be involved, the second possibility appears more pertinent in the present study, since the anaerobic condition would be too late, in time, to prevent a highly activated radiation product from reacting with the cytochromes, but it could prevent the affected cytochromes from reacting further.

There is, however, a function more important than holding the cytochromes in abeyance (Bachofer, 1956). It appears that checking aerobic metabolism permits anaerobic metabolism to restore essential molecules needed for normal development. The fact that recovery was greater for eggs held anaerobically at 30° C. than at sub-optimal temperatures indicates that anaerobic metabolism is associated with the recovery under consideration.

Summary

1. X-irradiation of *Ascaris lumbricoides suum* eggs produced delay of cell cleavage and reduced the percentage of eggs that completed embryogenesis. The time required for cleavage of irradiated eggs was reduced by an anaerobic treatment after

irradiation. The percentage of eggs that completed embryogenesis was increased by the same post-irradiation anaerobiosis. After the anaerobic treatment, eggs must be incubated aerobically since there is no perceptible development under anaerobiosis, although recovery takes place during this period. This recovery is greater at 30° C. than at sub-optimal temperatures.

2. Maximum recovery was obtained for eggs placed immediately after irradiation under anaerobiosis for periods of approximately 15 hours or more at 30° C. If the anaerobic treatment is delayed for 15 hours, the recovery is negligible.

3. Post-irradiation treatment with cyanide also fostered recovery from x-irradiation comparable to that secured with anaerobiosis.

4. Recovery was not due to delay of cleavage: the critical period for recovery took place long before cell division occurred even in air-saturated non-irradiated controls.

5. A cytochrome system in the eggs was demonstrated. The effects of cyanide treatment and anaerobiosis suggest that the mechanism of recovery may involve inhibition of the cytochrome system, which is prevented from participation in the reactions producing the expected deleterious effects of irradiation. There is, however, a positive contribution attributable to anaerobic metabolism, since recovery is greatest at optimal temperatures under anaerobiosis.

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RADIOCOBALT ACCUMULATION IN TETRAHYMENA

JOHN V. SLATER

Dept. of Biology, University of Buffalo, Buffalo 14, N. Y., and Biology Division, Oak Ridge National Laboratory [1]

Previous studies have indicated that cobalt is essential for growth in *Tetrahymena* (Slater, 1952; Roth, 1956), but there is no information available about cation uptake in this animal. Although the major function of cobalt is believed to be that of serving as part of the vitamin B_{12} molecule (Marston, 1952), cobalt is also implicated as the element *per se* in important hydrolytic reactions (Johnson and Berger, 1942).

The present experiments were designed to study the accumulation of cobalt in protozoans during growth. Elucidation of some of the factors regulating cobalt transport between the medium and the organism was also attempted. This study included the growth phase, the influence of deficient medium on exchange, the influence of population density on uptake per animal, and the effect of ion concentration on uptake.

Materials and Methods

Strain E of *Tetrahymena pyriformis* was used in this investigation, and all the experiments were performed in synthetic medium (Slater, 1952). Calcium, uracil, and adenylic acid were omitted from the media in all instances and cytidylic and guanylic acids were reduced to 10 μgm./ml. levels. In some experiments, growth effects were eliminated by use of media deficient in essential growth factors.

Cobalt-60 was used as a tracer and adjusted to 0.1 μc./ml. (final concentration) except where indicated. Cultures were grown in 10 ml. of synthetic medium in 18-mm. Pyrex tubes, and growth was measured turbidimetrically with a Lumetron (Model 400) colorimeter equipped with a red (650 mμ) filter. Radiations were detected with a deep-well scintillation detector and a Nuclear Instrument and Chemical Corporation Scaler (No. 162). Constriction chamber centrifuge tubes enabled clear separations of organisms from supernatant upon mild (100 G, one minute) centrifugation. The culture was washed with non-radioactive synthetic medium to remove excess fluid. The histidine in this medium is known to form a strong complex with cobalt (Burk *et al.*, 1946).

Prior to the introduction of Co^{60}, no cobalt was detected in the synthetic medium by ultraviolet emission spectroscopy, the porous cup technique being used. One

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liter of synthetic medium was concentrated 500-fold by evaporation for this analysis, and it was estimated that less than 0.01 μgm./ml. of cobalt ion was present.

Radiation effects from the tracer used have, in many instances, been known to influence physiological processes. The extreme resistance of *Tetrahymena* to radiation (Elliott and Slater, 1951), however, makes it unlikely that any influence from the tracer's radiation was significant during these experiments.

After the protozoans were separated from the supernatant by centrifugation, they were placed in two-ml. volumetric tubes and adjusted by micropipettes to 2.0-ml. volumes with distilled water. They were then transferred quantitatively to

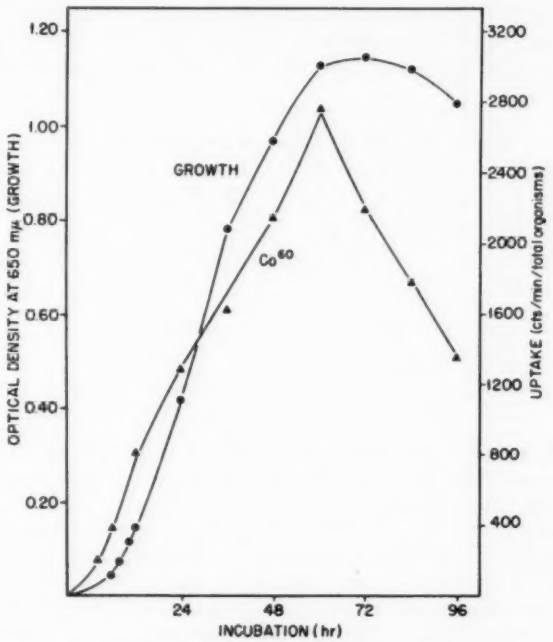

FIGURE 1. Cobalt-60 uptake and release during growth in *Tetrahymena*.

plastic tubes. Since the volume of the column of radioactive substance materially affected the number of counts registered by the scintillation detector, exactly 2.0-ml. volume adjustments were used throughout.

RESULTS

1. Uptake during growth

In the first series of experiments, Co^{60} at 0.01 μc./ml. (final concentration) was introduced into each culture at the beginning of the experiment as a tracer for the movement of this element during growth. Spectrographic analysis revealed that the

added cobalt amounted to 3.7 μgm./ml. (final concentration). No growth effects were noticed from cobalt at this concentration in preliminary experiments.

There was a steady uptake of cobalt during growth and an abrupt release of this ion shortly after the stationary phase was reached (Fig. 1). The temperature in the typical experiment reported was 27.5° ± 0.5° C., and the initial inoculum from mid-log phase cultures amounted to 185,000 ± 5% animals per tube. The total uptake during 60 hours of growth amounted to 0.97 μgm. of Co per total mass of cells, or about 26% of the cobalt present. This amounted to 4.6×10^{-8}% of the total cobalt present per hour per organism. Further calculations revealed that each *Tetrahymena* at 60 hours possessed about 10^9 atoms of cobalt. The number of animals remained constant from the sixth to the twelfth hour after inoculation (Table I) although the optical density measurements increased steadily. Uptake of cobalt during this period was probably associated with the increase in volume of the individual

TABLE I

Cobalt-60 uptake during growth for the first 12 hours

Time (hr.)	Optical density at 650 mμ	Number of animals	Uptake for total population (cts./min.)
0		185,000	
¼			77
2			208
4			252
6	0.04	479,000	381
8	0.07	459,000	489
10	0.11	457,000	743
12	0.14	466,000	806

organism. The volume of *Tetrahymena* reaches a maximum near the upper third of the log phase and falls off to about one-half this value upon reaching the stationary phase (Slater and Elliott, 1951).

The release of Co^{60} during the stationary phase was studied for only two days to avoid the possibility of measuring cobalt release from disintegrating cells. Microscopic observation of the protozoans during this period failed to reveal any obvious morphological breakdown, although an imperceptible physiological breakdown is certainly not an impossibility. Fifty per cent of the accumulated cobalt was released into the medium in 36 hours with a rate amounting to 1.4×10^{-6}%/hour/organism.

2. Effect of number of animals on uptake

The influence of number of animals on uptake per animal was studied. Eight-hour periods of time were selected to minimize growth effects and any influence from the accumulation of metabolic wastes. The number of animals present had a definite effect on uptake (Fig. 2) per animal. Populations of the order of 10^4 animals became nearly ten times as radioactive as populations of 2×10^6 organisms. At these high population densities it is not improbable that there was a great deal of competition for oxygen and also that harmful metabolic wastes resulted in inhibitory effects. Population densities of 2×10^5 cells, however, were far from being crowded under the experimental conditions and yet contained only one-third the ac-

tivity of the lowest concentration. In the experiment shown, the temperature was maintained at 26.5° ± 0.5° C., and cobalt was introduced at the 0.01 μc./ml. level.

3. Cobalt release in deficient medium

Release of cobalt was studied in media deficient in essential growth factors, salts, and glucose. Log-phase cultures were allowed to incubate initially in complete synthetic medium containing 0.01 μc./ml. of Co^{60} for 24 hours. The animals were

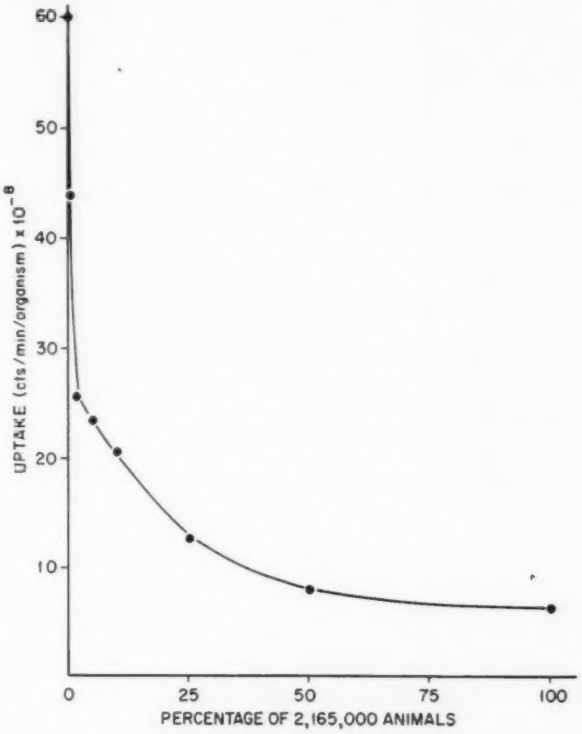

FIGURE 2. Influence of number of animals on uptake per animal.

then removed by centrifugation, washed once with deficient medium, and resuspended in deficient medium. This medium was used to prevent growth effects. In the typical experiment illustrated (Fig. 3) the initial population amounted to 415,000 cells/tube. These had grown to about twice this number at the time of introduction to the "cold" medium.

Two mechanisms are evident during cobalt release under these conditions. The first is very rapid and takes place within two hours. The rate of release during this time was 13%/hour/organism. The second mechanism is much slower and

amounts to 1.7%/hour/organism. Under these conditions, 50% of the cobalt was released in about 20 hours.

4. Cobalt concentration ability

The ability of protoplasm to concentrate certain elements has been known for a long time. *Tetrahymena* is no exception in this capacity. In the first series of experiments, uptake in relation to cobalt concentration was studied during 12 hours.

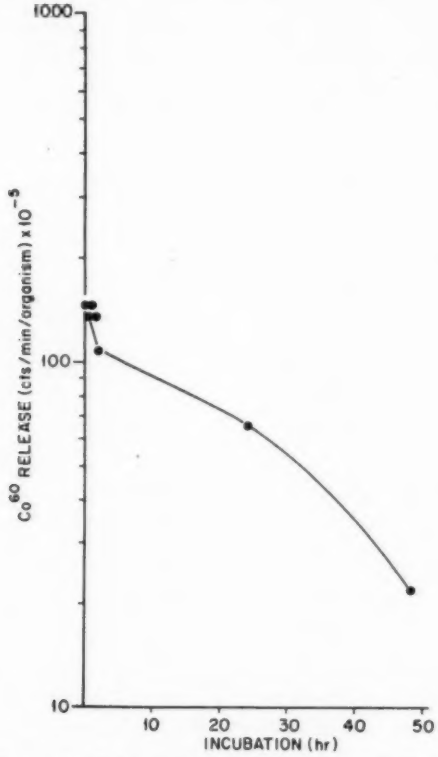

FIGURE 3. Release of cobalt-60 in deficient medium.

Complete synthetic medium was used in all instances, and the relatively short time interval was used to minimize growth effects. The concentrations used varied from 0.0005 μc./ml. of Co^{60} (3×10^{-6} *M*) to 0.0100 μc./ml. of Co^{60} (6×10^{-5} *M*). Uptake during 12 hours' incubation was found to be directly proportional to concentration (Fig. 4). The animals concentrated cobalt to the extent of 4.4–5.7 times that present in similar volumes of the environment regardless of the amount of isotope present (Table II). The volumes of individual animals used in these calculations are adapted from data presented earlier (Slater, 1951).

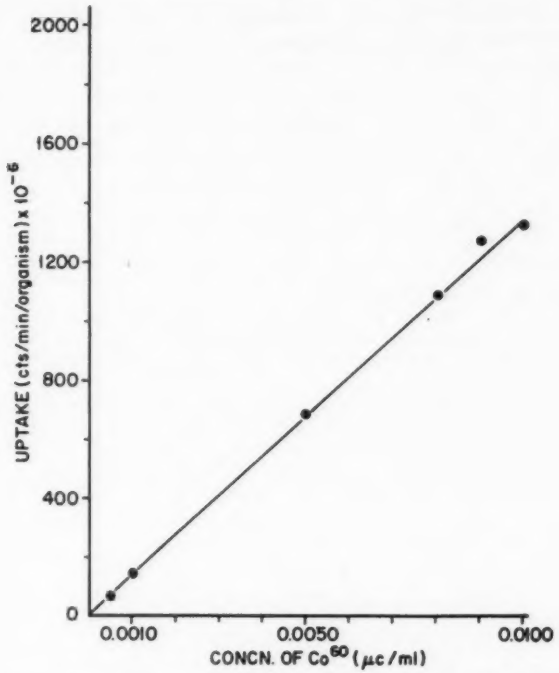

FIGURE 4. Effect of cobalt-60 concentration on 12-hour uptake.

TABLE II

Influence of cobalt concentration on uptake in 12 hours

Conc. of Co^{60} (μc./ml.)	Co^{++} (μgm./ml.)	Cts./min./total population	Cts./min./vol. occupied by population*	Degree of conc.** cts./min./total pop. / cts./min./vol. occup.
0.0100	3.70	1104	221	4.9
0.0098	3.63	942	216	4.4
0.0096	3.55	1211	212	5.7
0.0094	3.48	1126	207	5.4
0.0092	3.40	1169	203	5.7
0.0090	3.33	1062	198	5.4
0.0080	2.96	910	176	5.2
0.0050	1.85	573	110	5.1
0.0010	0.37	117	22	5.3
0.0005	0.185	58	11	5.3

* Calculated from μc./ml. times cts./min./μc. times volume occupied by protozoans after 12 hours growth. One ml. containing 0.01 μc. of Co^{60} gives 10,500 cts./min. The volume occupied by these populations equaled 0.0212 ml. after 12 hours growth.

** Representing the concentration of cobalt by the entire population of animals over that contained in comparable volumes.

TABLE III

Influence of cobalt concentration on maximum uptake

Conc. of Co^{60} (μc./ml.)	Co^{++} (μgm./ml.)	Max. uptake cts./min./total population	Cts./min./vol. occupied by population*	Degree of conc. cts./min./total pop. / cts./min./vol. occup.
0.01	0.764	2064	265	7.8
0.005	0.508	1373	132	10.4
0.001	0.148	399	27	14.8
0.0005	0.061	165	13	12.7
0.0002	0.025	68	5	13.6

pH, 7.4; temperature, 25.7° ± 0.5° C.

* Calculated from μc./ml. times cts./min./μc. times volume occupied by protozoans at maximum uptake. One ml. containing 0.01 μc. of Co^{60} gives 10,500 cts./min. The volume occupied by these populations equaled 0.0253 ml. of maximum uptake (Slater, 1951).

The influence of cobalt concentration on maximum uptake during growth was also studied. The greatest absolute amount of this ion was taken up by cultures in the presence of 0.764 μgm./ml. of Co^{++} (Table III). The ability to concentrate this ion over that contained in the medium, though, reached maximum at 0.1–0.5

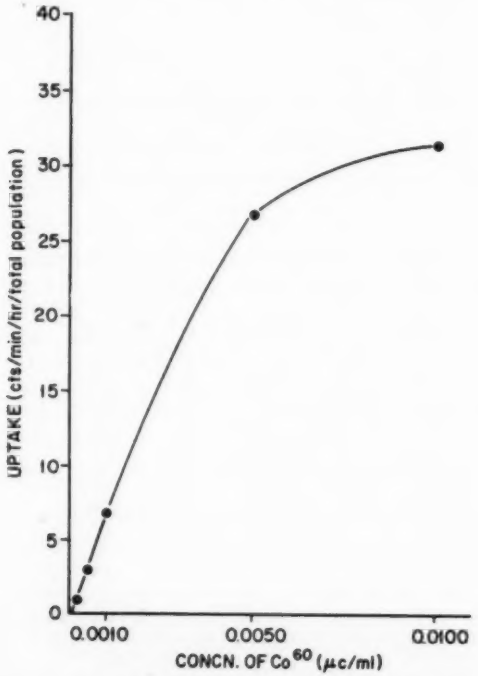

FIGURE 5. Uptake of cobalt-60 with concentration at 36 hours.

μgm./ml. of Co^{++}. The maximum uptake was about one and three quarters times that with 0.764 μgm./ml. of Co^{++}. Correspondingly, the rate of uptake seemed to be linear with concentrations up to 0.508 μgm./ml. of Co^{++} (Fig. 5) but was less at higher concentrations.

Discussion

The uptake of cations by animal cells involves a complex spectrum of interrelated processes, any one of which may alter the total cellular absorptive capacity. Among these controlling factors are the rate of utilization and probably intracellular translocation. Probably the uptake of inorganic substances first involves a combination with organic cell constituents (Sutcliff, 1954); specific metabolic pumps may also be involved. These cations then may become distributed to different cellular sites in response to varying chelation forces, which appear as the environmental mixture of cations changes. Thus during periods of major synthesis and growth, forces primarily concerned with cell divisions and external membrane changes might be most active, while during periods of relative inactivity, forces concerned with exchange, turnover, and simple release might well become more active. Chelation forces may also be involved in mitochondrial physiology and are probably involved in ion transport in these particulates. Studies of the relative binding abilities of various sites within the cell must certainly be done if the intricacies of cation transport and translocation are to be elucidated.

In *Tetrahymena*, it was shown that the population density may greatly influence the uptake of cobalt per cell. At great densities, cellular chelate forces may compete for environmental cations. It is also considered likely that competition for dissolved nutrients in general may inhibit the transport mechanism for any given cation.

It was demonstrated earlier that cobalt was essential for growth in purified synthetic medium without glucose. The physiological role of this cation in protozoans where an extraneous source of the B_{12} molecule is not required is not clear. It has been suggested that cobalt ion in these experiments may act non-specifically to increase the availability of other cations by releasing them or displacing them from complexes (Ford and Hutner, 1955, pp. 101–136) at the cell surface (Hutner *et al.*, 1950). Since Roth (1956) has shown that nickel does not have the same effect as cobalt on growth in *Tetrahymena*, and since the present experiments have demonstrated that cobalt is differentially bound to the animal, depending on the concentration of the cation, any non-specific effect may be minimal.

The uptake of cobalt during growth has been studied in *Bacillus subtilis* (Tanaka *et al.*, 1952), *Neurospora crassa* (Ballentine and Stephens, 1951), and *Saccharomyces cerevisiae* (Nickerson and Zerahn, 1949). No studies involving protozoan uptake of this ion seem to have been published.

Sizable losses of cobalt were observed with advancing age of the population in all the above organisms. In both *Tetrahymena* and *B. subtilis*, the release of the ion began abruptly after the stationary phase began. A comparison of the concentrating abilities of *Saccharomyces* and *Tetrahymena* showed that *Saccharomyces* had nearly 70 times the ability to concentrate cobalt possessed by the protozoan. Two washes of *Tetrahymena* did not appreciably remove the ion, and over 20 hours' washing of *Saccharomyces* also showed that cobalt was firmly held. Nickerson and Zerahn (1949) suggested that the presence of peripherally located metaphosphate might be significant in the accumulation of "metals" from dilute solution by yeasts.

In *Neurospora* (Ballentine and Stephens, 1951), at least 40% of the cobalt accumulated was present in stable cobalto-proteins. Fractionation of these compounds revealed the presence of a soluble fraction comprising 57% of the stably bound cobalt and a nearly submicroscopic particulate fraction. Similar cobalto-proteins were also found in *Chlorella vulgaris* and in the leaves of the musk melon and tomato. As with *Neurospora, Saccharomyces,* and *B. subtilis, Tetrahymena* concentrated cobalt against a concentration gradient.

Scott and Ericson (1955) reported that cobalt was absorbed by the marine alga, *Rhodymenia palmata,* and became bound within the plant as a complex quite different from B_{12}. Analysis of this complex revealed the presence of several components. Thus cobalt may play a multiphysiological role in protoplasm. Ericson (1952) reported that sea weed possessed an unusual ability to absorb and concentrate Co^{60} but the absorption of vitamin B_{12} was very limited and could not account for the concentration of the cation. In earlier work on the essentiality of cobalt for growth in *Tetrahymena* (Slater, 1952), it was shown that a definite response could be obtained in purified synthetic medium when as little as 0.5 μgm./ml. of cobalt ion was present. Under the conditions of those experiments, this amount of cobalt was equivalent to about 5×10^{10} atoms of cobalt per animal. In the present experiments, nearly 10^9 atoms of cobalt were accumulated per animal, presumably for non-growth purposes since growth progressed in controls without added cobalt. Thus, in purified synthetic medium, when extraneous cations are removed to a large degree, nearly 50 times as much cobalt is used for growth as is accumulated when growth proceeds under the influence of other cations.

In a study on cobalt localization in pooled white mouse cells, Rosenfeld and Tobias (1951) reported that most of the element was present in the cytoplasm and about 1% of it was firmly bound to cellular protein. Very little was discovered in the nuclei. Most of the cytoplasmic association was with the globulin in the bound fraction. The slowly released fraction of cobalt in *Tetrahymena* (Fig. 3) may be associated with a firmly bound fraction of this type, but this fraction appears to be about 75% of the total. The intracellular particulate localization of cobalt remains to be elucidated.

Summary

1. A steady uptake of radioactive cobalt was observed during the growth of *Tetrahymena* in synthetic medium. When the initially-added amount of cobalt was 3.7 μgm./ml., nearly 26% of the total was accumulated during 60 hours of growth. This amounted to approximately one billion atoms of cobalt per animal.

2. Upon reaching the stationary phase, sizable amounts of cobalt were released from the population. Fifty per cent release was observed in 36 hours with a rate amounting to 1.4×10^{-6}%/hour/organism.

3. The number of animals present had a definite effect on the accumulation of cobalt per animal. Populations of 10^4 animals became nearly ten times as radioactive as populations containing two million organisms.

4. In nutritionally-deficient media, the release of cobalt was biphasic. The first was very rapid and took place within two hours. The initial rate of release of cobalt amounted to 13%/hour/organism. The second mechanism was much slower and amounted to 1.7%/hour/organism. Fifty per cent release was noticed in 20 hours under these conditions.

5. As the concentration of cobalt was varied, the rates of uptake increased rapidly. Concentrations higher than 1.85 μgm./ml. had little effect. The greatest absolute amount of cobalt was accumulated when 3.7 μgm./ml. was initially present in the medium, but the ability to concentrate this ion over that contained in the environment reached a peak at 0.37 μgm./ml. of cobalt ion.

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CONTRIBUTIONS TO SURVIVAL MADE BY BODY CELLS OF GENETICALLY DIFFERENTIATED STRAINS OF MICE FOLLOWING X-IRRADIATIONS[1]

JANICE STADLER AND JOHN W. GOWEN

Department of Genetics, Iowa State College, Ames, Iowa

Our strains of inbred mice have inherited relatively stable differences in their sensitivities to absorbed radiant energy (Gowen, 1950; Gowen and Zelle, 1945; Gowen and Stadler, 1956; Grahn, 1954) from whole-body irradiation. These differences are related to fixed variations in the normal cell structures of the strains (Gowen, 1945; Gowen and Calhoun, 1943; Gowen, 1952; Weir, 1949). Progress in understanding the mechanisms of irradiation damage may be advanced if genetic differences in resistance are traced to the cells as a whole or to particular organ systems. Regional body irradiation affords a technique for localizing organs significant to radiation resistance. This technique along with surgical removal and/or shielding before organ exposure has been tried with some indications that given organs are significant to irradiation resistance. Examination of irradiation effects on genetic strains of mice having known ranges in resistance combined with partial body exposure without confounding by surgical interference offers a promising means of attacking this problem.

Materials and Methods

The host constitutions in these experiments were differentiated into 5 distinct lines through 30 or more generations of brother-by-sister matings accompanied by selection for specific inherited types. The strains are homozygous albino but differ in coat color at the agouti locus. They are differentiated for resistance to *Salmonella typhimurium*, the typhoid-causing bacteria of mice. Under comparable conditions over a period of more than 20 years the mice of these strains maintain their relative resistances to 200,000 organisms to that observed in these experiments, S 100%, Z 45%, K 39%, Q 0% and Balb/Gw, hereafter called Ba, 0%. The strains differ genetically in body weight, growth rate, heart, kidney, liver, spleen and testis weights, serum globulins, leucocyte number, fixed phagocytic cells, macrophages, and cells metabolizing fat and storing glycogen as well as other significant physiological characteristics. The environment of the animals throughout the experimental period, as well as through many breeding generations, was that of a controlled laboratory where feed, water and management were uniform.

All mice were 46 ± 3 days of age at the time of irradiation. Strains and sexes were randomly distributed across the different treatments. Thirty-seven weeks

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were required to completely fill the experiment as designed. A General Electric Maxitron operated at 250 pkv, 30 ma with 0.25 mm. Cu + 1 mm. Al filtration at a distance of 47.5 cm. from anode to mid-mouse was used for the x-irradiation of all mice. The dose rate varied from 160 r/minute to 180 r/minute. Part of this range was accounted for by a change of x-ray tubes. Mice were held for x-irradiation in perforated plastic tubes with cork stoppers. These tubes were arranged in a wooden rack in two rows of eight tubes placed side by side with the closed ends of each row facing each other. The field of radiation covered by this rack of tubes permitted a range of only 12 r per minute over the entire area.

The experiment performed and analyzed was designed as a factorial having four elements. Five inbred strains of mice having known differences in x-ray and typhoid sensitivities were utilized in comparable numbers. The numbers for the two sexes were balanced for each strain. There were four x-ray exposure doses: 0, 320, 480 and 640 roentgens. The levels of x-ray dosage were chosen to span the range from no effect to nearly complete lethality when the mice were exposed to whole-body irradiation.

There were eight combinations of body coverage or exposure. The body of the mouse was divided into three regions—head, mid and rear. Shielding was with $\frac{1}{8}$ inch-thick strips of lead laid across the tubes covering the region or regions of the mice for a given treatment. The eight combinations of regions exposed are shown below.

Region Exposed

None
Head
Mid
Rear
Head-Mid
Head-Rear
Mid-Rear
Whole-Body

The head region or anterior third of the body extended into the thorax. The middle third of the body, mid region, included that part of the body containing lower thorax, and abdominal cavity containing stomach and upper intestinal tract, liver, spleen, adrenals, ovaries and kidneys. The posterior third of the body, rear region, included the lower intestinal tract, bladder, and urinary system and testes of the males. These regions are illustrated in Plate 1.

The eight groups of different regional exposures were treated with 320 r, 480 r and 640 r making a total of 24 different x-ray treatment groups. In addition the mice of one group were put in tubes and completely lead-covered for a time comparable to that of the longest dose, 640 r, but were not exposed. This group acted as a control on handling as well as for un-irradiated, 0 r group.

There were 25 treatment groups with 5 strains and 2 sexes making up 250 cells in the experiment. Each cell represented a different strain, sex, and treatment. A minimum of 25 mice were treated in each cell. Some cells contained a few extra animals. The completed experiment involved a total of 6904 mice.

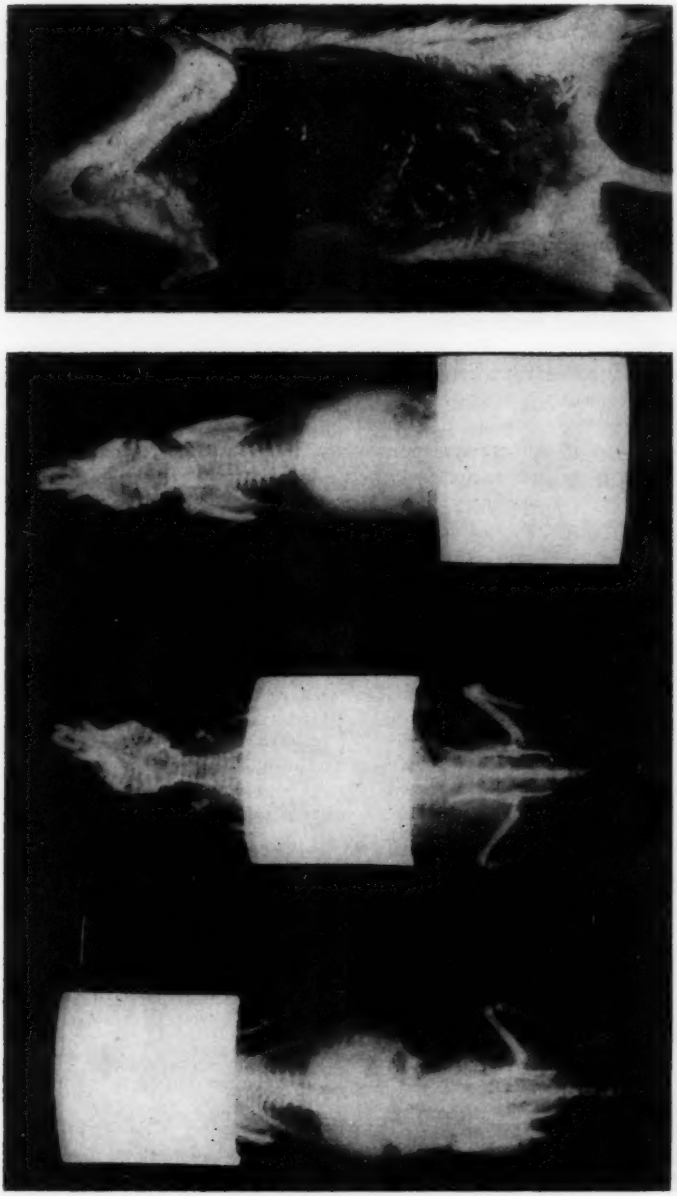

PLATE 1. Radiographs showing shielding marking off the three regions and their relation to the anatomy of the mouse.

The immediate effects of x-ray were largely completed 12 days following irradiation. Although there were marked differences in survival times between the different strains, few deaths occurred after the twelfth day. A 15-day interval following irradiation was allowed to cover the direct effects of exposure. Deaths were recorded daily and survivors of the fifteenth day were considered as surviving mice.

The ⅛-inch lead sheets used to delimit the body regions exposed to irradiation protected the shielded regions from the greater portion of irradiation. But this protection was not complete. The ⅛-inch lead was sufficient to absorb all but 0.75 r per minute. The back scattering of the radiation into the shielded regions accounted for larger doses of radiant energy in the protected regions. Measurements made when the tubes were placed on a nylon mesh screen under the same conditions showed that the back scattering largely came from the wooden rack used to hold the plastic tubes containing the mice. Only 7.4 r or 1.2 per cent of the 640 r dose was absorbed in the lead-covered mid-region when head and rear regions were exposed, whereas 78 r or 12.2 per cent of the x-rays was absorbed when the wooden rack was used. The amount of radiation absorbed in the different shielded regions varied from 3 to 12.2 per cent of the dose given. The region receiving most scattered radiation was the mid-region when the head and rear regions were exposed. This could be expected as the scattering could enter from two directions. The amount of radiation absorbed in any shielded region should tend to magnify somewhat the effects of the different treatments and reduce the differences between the treatments. The effects of scattered radiation were distributed through all treatment groups and tended to balance from one treatment to another. That this amount of radiation absorbed into the protected regions could lessen in some degree the recovery potentials of the mice treated in the region is recognized. The total roentgens scattered and absorbed should make this factor a minor contributor to the total variation.

The results of localizing radiation to particular regions of the body have been determined by comparison of sexes, strains and dosages across all regional exposure treatments in the factorially designed experiment. Two separate criteria have been used to measure the effects of irradiation, the percentage survival of the treated mice and the length of survival in days within the observational period.

Because of the unequal numbers of mice in the different treatment groups, all data have been analyzed throughout this paper by using disproportionate frequency analyses. Binomial analyses were used for the survival data and the customary methods for mean length of survival within the observational periods.

The data based on percentage survival did not allow for the full expression of the differences between strains, etc. Survival range had fixed limits at 0 and 100 per cent. These limits confined the quantitative estimates of the effects, *i.e.*, strain or radiation, to values within this range. Consequently all conclusions drawn from the percentage survival data led to minimal estimates by the nature of these limitations of the scale.

The data on length of survival were confined by one limiting measurement, the total length of the observation period. This fixed measurement again limited the full expression of the effects of irradiation on the mice. Any differences observed in both sets of data for each response were therefore minimal estimates of these consequences following the different treatments.

Experimental Results

The main purpose of this experiment was to examine the effects of irradiation to particular organs (body regions) and to their summation of effects on the cells of the body as a whole. Two variables are intrinsic in the data. Known genetic differences may assist in these analyses by introducing reliable differences in the resistance levels of the mouse strains. Sex of the mice utilized in the tests could affect the results although previous experience has shown that this is a minor factor in the expression of the mouse typhoid syndrome. The effects of sex and strain will be analyzed separately. The sex effects are considered first.

Effects of Sex

Irradiation may cause acute effects leading to death. In the range of x-ray exposures of this experiment these radiation deaths were largely over by 12 days post-irradiation. Consequences of the x-ray treatments were measured by percentages

Table I

Effect of sex on radiation sensitivity. Per cent survival in 15 days

Dose (r)	Region exposed	Mean per cent survived		Variance analyses			
				Within sex		Between sex 1 d.f.	
		Male	Female	d.f.	M.S.	M.S.	F
0	None	100	100	289	—	—	—
320	None	100	99	266	.004	.004	1.0
	Head	100	100	270	—	—	—
	Mid	100	99	267	.004	.004	1.0
	Rear	100	100	268	—	—	—
	Head-mid	99	100	270	.004	.004	1.0
	Head-rear	100	100	267	—	—	—
	Mid-rear	99	100	268	.007	.015	2.0
	Whole-body	97	95	271	.035	.019	0.5
480	None	98	100	257	.011	.034	3.0
	Head	100	100	258	—	—	—
	Mid	99	98	269	.018	.003	0.2
	Rear	100	100	265	—	—	—
	Head-mid	99	100	263	.004	.004	1.0
	Head-rear	100	100	269	—	—	—
	Mid-rear	99	100	276	.007	.015	2.1
	Whole-body	59	62	284	.241	.062	0.3
640	None	100	100	279	—	—	—
	Head	99	99	295	.010	.004	0.4
	Mid	97	97	286	.027	.000	—
	Rear	99	100	289	.003	.004	1.0
	Head-mid	92	95	284	.062	.092	1.5
	Head-rear	99	99	286	.010	.003	0.3
	Mid-rear	80	81	287	.159	.005	0.0
	Whole-body	7	3	271	.045	.090	2.0

TABLE II

Effect of sex on radiation sensitivity. Mean days survived in 15-day period

Dose (r)	Region exposed	Mean days survived		Variance analyses			
				Within sex		Between sex 1 d.f.	
		Male	Female	d.f.	M.S.	M.S.	F
0	None	15.0	15.0	289	—	—	—
320	None	15.0	15.0	266	0.06	0.06	1.0
	Head	15.0	15.0	270	—	—	—
	Mid	15.0	15.0	267	0.00	0.00	1.0
	Rear	15.0	15.0	268	—	—	—
	Head-mid	15.0	15.0	270	0.06	0.06	1.0
	Head-rear	15.0	15.0	267	—	—	—
	Mid-rear	14.9	15.0	268	0.15	0.30	2.0
	Whole-body	14.9	14.8	271	1.43	0.39	0.3
480	None	14.9	15.0	257	2.90	0.75	0.3
	Head	15.0	15.0	258	—	—	—
	Mid	14.9	14.8	269	1.20	1.02	0.9
	Rear	15.0	15.0	265	—	—	—
	Head-mid	15.0	15.0	263	0.14	0.13	1.0
	Head-rear	15.0	15.0	269	—	—	—
	Mid-rear	14.9	15.0	276	0.52	0.93	1.8
	Whole-body	13.6	13.8	284	5.02	1.91	0.4
640	None	15.0	15.0	279	—	—	—
	Head	14.9	14.9	295	0.38	0.06	0.2
	Mid	14.8	14.7	286	2.34	0.09	0.0
	Rear	14.9	15.0	289	0.28	0.28	1.0
	Head-mid	14.2	14.6	284	5.00	8.79	1.8
	Head-rear	15.0	15.0	286	0.06	0.03	0.5
	Mid-rear	13.2	13.3	287	14.18	0.42	0.0
	Whole-body	9.4	8.9	271	9.52	12.70	1.3

of the animals which survived for 15 days following exposure and by the average lengths of survival of the irradiated groups. The data are subdivided for each sex into the x-ray dose and the region of the body in which the mouse received the irradiation. The strains were nearly balanced in numbers and were combined in these tests.

Table I gives the data on the mice which survived the different x-ray treatments. The first column presents the x-ray dosage in air at the mid point of the mouse's body. Column 2 lists the body regions exposed to x-rays. Columns 3 and 4 give the mean survival as per cent of the mice surviving 15 days after the x-ray exposure according to the sex. The variance analyses give the degrees of freedom (d.f.) for the variances of the mice within sexes, the mean square (M.S.) between sexes with 1 d.f. and the F values for the mean squares. Throughout this paper * shows significance at the 0.05 level and ** at the 0.01 level.

Table I shows that only animals which received whole-body irradiation had appreciable mortality. In the 15-day period no mice died in the untreated group; the

320 r whole-body exposed mice showed a few scattered deaths, 3 and 5 per cent; the 480 r dose was more lethal, about 40 per cent of the mice died; and the 640 r dose was almost completely lethal, only 3 and 7 per cent survived of the 273 mice treated. Consideration of sex effects on radiation survival was consequently almost entirely limited to the 480 r and 640 r whole-body irradiation groups. The differences in responses of the sexes to irradiation were small throughout the 25 comparisons. In no case was a significant difference observed. The 480 r and 640 r whole-body exposures were severe enough to test any real biological changes in the physiologies of the sexes suffered through irradiation exposure. No sex differences appeared. In view of these facts the data for sexes have been combined in further consideration of these experiments on survival to x-radiation.

The severity of the x-ray effects may be measured by the number of days a mouse survives following x-ray exposure. Table II gives these data in the same form as Table I, with columns 3 and 4 showing the average number of days the males and females survived of the 15 days subsequent to x-irradiation. All treatment groups except the whole-body exposures at 320 r, 480 r and 640 r showed practically complete survival. The mean day survivals for whole-body exposures were 0 r—15 days, 320 r—14.9 days, 480 r—13.7 days and 640 r—9.1 days. The mean length of survival of the mice to whole-body irradiation was but slightly reduced by 320 r, was lowered by 480 r and was severely reduced by 640 r. The tests for sex effects on length of survival were only critical for the 480 r and 640 r whole-body irradiation groups. The sexes showed comparable mean days of survival. In no case was there a significant difference between sexes.

These results further support the conclusion that the sexes in mice are equally affected by x-irradiation. The conclusions on x-ray effects which are derived from these data will not be altered by combining the observations of the two sexes.

These tests also furnish a measure of the degree of protection afforded the animals by the coverage with lead plates. Animals of 0 r group were not exposed to x-rays but the bodies of the mice were completely shielded by ⅛-inch lead plates. The unexposed and 640 r groups had 100 per cent survival. In the 320 r treatment group exposed and lead-shielded, one female out of 268 animals died; the 480 r group had three males out of 259 mice die. Mortality at the three dose levels appeared fortuitous and unrelated to the irradiation. It was concluded that the protection with the lead shields was adequate for all groups.

Genetic Effects of Strains

The mice utilized in this experiment were known to exhibit differences in their abilities to withstand radiation. These differences have become isolated into different strains. The data on the genetic effects on resistance to radiation as evidenced by different strains within a treatment are presented in Table III.

Five treatments displayed strain effects on x-ray sensitivity. These exposures were: whole-body at 320 r and 480 r and mid, head-mid and mid-rear regions at 640 r. Examination of the mean strain survivals indicates that these differences came largely from the high susceptibility of the Ba strain. All strains were affected by the whole-body exposures, and the severity increased with dose. At 640 r the effects were so severe as to have overreached strain differences as judged by the F test, even though the strains still retained their relative order of resistance.

TABLE III

Genetic effects of strain differences on radiation sensitivity. Per cent survived 15 days post-irradiation

Dose (r)	Region exposed	Strains Per cent survival					Variance analyses			
							Within strains		Between strains 4 d.f.	
		S	Z	K	Q	Ba	d.f.	M.S.	M.S.	F
0	None	100	100	100	100	100	286	—	—	—
320	None	100	98	100	100	100	263	.004	0.004	1.1
	Head	100	100	100	100	100	267	—	—	—
	Mid	100	100	100	100	98	264	.004	0.004	1.0
	Rear	100	100	100	100	100	265	—	—	—
	Head-mid	100	100	98	100	100	267	.004	0.004	1.0
	Head-rear	100	100	100	100	100	264	—	—	—
	Mid-rear	100	100	100	100	97	265	.007	0.014	1.9
	Whole-body	98	100	94	100	90	268	.034	0.107	3.2*
480	None	100	100	96	100	98	254	.011	0.015	1.3
	Head	100	100	100	100	100	255	—	—	—
	Mid	98	100	98	98	97	266	.018	0.008	0.5
	Rear	100	100	100	100	100	262	—	—	—
	Head-mid	100	98	100	100	100	260	.004	0.004	1.0
	Head-rear	100	100	100	100	100	266	—	—	—
	Mid-rear	100	100	100	100	97	273	.007	0.013	1.9
	Whole-body	79	75	54	66	31	281	.213	2.116	9.9**
640	None	100	100	100	100	100	276	—	—	—
	Head	98	100	100	98	98	292	.010	0.005	0.5
	Mid	100	100	95	100	92	283	.026	0.082	3.1*
	Rear	100	100	100	100	98	286	.003	0.003	1.0
	Head-mid	98	98	96	100	75	281	.054	0.633	11.7**
	Head-rear	100	100	98	98	98	283	.010	0.006	0.5
	Mid-rear	92	96	71	98	49	284	.124	2.624	21.1**
	Whole-body	11	4	4	6	0	268	.045	0.085	1.9

Strain differences were separated most clearly when the animals were exposed to the 640 r. These strain differences appeared in all treatments. The 480 r treated mice showed the strain effects only in the whole-body irradiated class. The 320 r treated groups were at the border line where a reduction in survival was only beginning to appear in the whole-body treated mice of the most susceptible strain. The whole-body 480 r irradiations ordered the strains for resistance; the S was most resistant followed in order by the Z, Q, K and the most susceptible Ba.

In the 640 r dose range S, Z and Q strains were not very different in their responses. The Ba strain again showed the greatest radiation sensitivity with the K strain somewhat less sensitive than the Ba and noticeably less resistant than the other three strains.

The mean days of survival for the 15-day interval following irradiation and the analyses of the strain differences are presented in Table IV.

Table IV confirms the major features of Table III. Strain differences in resistance to irradiation were brought out in the 480 r whole-body treatment and with 640 r exposure when the mid, head-mid, mid-rear and whole body were treated. The significance tests identify a difference in radiation effects which made the two measures, per cent survival and length of survival, desirable. The 320 r whole-body exposure was sufficient to make the strain differences in per cent survival significant, whereas the mean length of survival did not show such differences. The mice that died, died late in the 15-day observation period. Following 640 r whole-body exposure, the mice showed only an insignificant difference in strain survivals even though all survival values were markedly reduced, whereas in length of survival the strain differences were accentuated to give a highly significant F value. The other three treatments, the mid, head-mid and mid-rear regions, suggest the mid region as the most sensitive to radiation damage.

TABLE IV

Genetic effects of strain differences on radiation sensitivity. Mean days survived in 15 days

Dose (r)	Region exposed	Strains Mean days survived					Variance analyses			
							Within strains		Between strains	
		S	Z	K	Q	Ba	d.f.	M.S.	M.S.	F
0	None	15.0	15.0	15.0	15.0	15.0	286	—	—	—
320	None	15.0	14.9	15.0	15.0	15.0	263	0.06	0.07	1.1
	Head	15.0	15.0	15.0	15.0	15.0	267	—	—	—
	Mid	15.0	15.0	15.0	15.0	15.0	264	0.00	0.00	1.0
	Rear	15.0	15.0	15.0	15.0	15.0	265	—	—	—
	Head-mid	15.0	15.0	14.9	15.0	15.0	267	0.06	0.06	1.0
	Head-rear	15.0	15.0	15.0	15.0	15.0	264	—	—	—
	Mid-rear	15.0	15.0	15.0	15.0	14.8	265	0.15	0.28	1.9
	Whole-body	14.8	15.0	14.9	15.0	14.5	268	1.41	2.30	1.6
480	None	15.0	15.0	14.8	15.0	15.0	254	0.29	0.52	1.8
	Head	15.0	15.0	15.0	15.0	15.0	255	—	—	—
	Mid	14.9	15.0	14.8	15.0	14.7	266	1.20	0.91	0.8
	Rear	15.0	15.0	15.0	15.0	15.0	262	—	—	—
	Head-mid	15.0	14.9	15.0	15.0	15.0	260	0.14	0.15	1.1
	Head-rear	15.0	15.0	15.0	15.0	15.0	266	—	—	—
	Mid-rear	15.0	15.0	15.0	15.0	14.7	273	0.52	0.84	1.6
	Whole-body	14.4	14.2	13.4	14.2	12.2	281	4.44	45.52	10.3**
640	None	15.0	15.0	15.0	15.0	15.0	276	—	—	—
	Head	14.9	15.0	15.0	14.9	14.9	292	0.38	0.20	0.5
	Mid	15.0	15.0	14.6	15.0	14.3	283	2.26	6.81	3.0*
	Rear	15.0	15.0	15.0	15.0	14.9	286	0.28	0.26	1.0
	Head-mid	14.9	14.8	14.8	15.0	12.8	281	4.32	53.74	12.4**
	Head-rear	15.0	15.0	15.0	15.0	15.0	283	0.06	0.03	0.5
	Mid-rear	14.4	14.7	12.4	14.8	10.2	284	11.01	235.54	21.4**
	Whole-body	10.6	10.4	8.8	10.4	5.4	268	5.72	265.42	46.4**

TABLE V

Effects of x-ray dosage to different body regions on survival 15 days after irradiation

Region exposed	Variance analyses							
	Doses 3 d.f.		Strains 4 d.f.		Dose × Strain 12 d.f.		Within dose and strain	
	M.S.	F	M.S.	F	M.S.	F	d.f.	M.S.
Percentage survival for 15 days post-irradiation								
None	No analysis—Practically all survived							
Head	No analysis—Practically all survived							
Mid	.048	3.9**	.044	3.6**	.017	1.4	1099	.012
Rear	No analysis—Practically all survived							
Head-mid	.291	18.6**	.156	10.0**	.162	10.3**	1094	.016
Head-rear	No analysis—Practically all survived							
Mid-rear	2.66	75.3**	.873	24.7**	.593	16.8**	1108	.035
Whole-body	54.00	734.9**	.869	11.8**	.480	6.5**	1104	.074
Mean days survived in 15 day post irradiation period								
None	No analysis—Practically all survived							
Head	No analysis—Practically all survived							
Mid	4.08	4.7**	3.27	3.7**	1.49	1.7	1099	.88
Rear	No analysis—Practically all survived							
Head-mid	22.12	19.1**	13.56	11.7**	13.46	11.6**	1094	1.16
Head-rear	No analysis—Practically all survived							
Mid-rear	220.15	73.7**	72.83	28.4**	54.61	18.3**	1108	2.99
Whole-body	2078.36	726.7**	131.31	45.9**	60.64	21.2**	1104	2.86

The mean days of survival of the five strains for the 25 different treatments showed somewhat less differentiation in the reactions of the strains to x-irradiation. The head-mid-rear exposure to 640 r showed the widest separation between Ba and K and these from the other three, S, Z, and Q strains. The order of the strains in resistance to radiation effects did not correspond to the order of these same strains in their natural resistance to mouse typhoid as noted earlier.

REGIONAL EFFECTS OF IRRADIATIONS AND GENOTYPES

Three elements were operating on the survival of the mice in this experiment, dosage of the x-rays, the region of the body exposed to x-rays and genotype as represented by strain of mice under treatment. Table V measures these effects first in terms of the mice surviving for 15 days following irradiation and second, the lower half of the table, in terms of mean length of survival within the 15-day period.

The data of Table V are presented for the body regions exposed, eight different categories in all. Of the eight groups four have not been analyzed as the deaths within these groups were few and scattered.

When the mid region only was exposed to x-rays, differences in dosage and in strains were evident and of equal significance. The dosage × strain interaction

was minor. Interaction between dosage × strain was a factor in survival when radiations were absorbed in the head-mid portion of the body. Both dosage and strain effects were large, with the dosage effects nearly double those of the strains. The interaction, however, was as large as the strain effects, indicating that the strains reacted differently to the different exposures.

X-rays to the rear two-thirds of the body gave even more noticeable effects. The effects of the dosages were markedly greater than the strain differences and the strain effects showed more than random deaths. The whole-body irradiations, those involving the head, mid and rear regions, were more severe than those of other treatments. The x-ray effects were so pronounced at the 640 r level (Table III) that they overshadowed the strain differences. The strain effects were clearer in the 480 r and 320 r whole-body treatment groups.

The data on length of survival within the 15-day interval following irradiation show essentially the same features as those for survival. Irradiation to the head-mid portion of the body was not as effective as that to the mid-rear portion, but both showed more change than when the x-rays were directed to the mid region alone. The mid region was sensitive but added irradiation to the rear or head regions increased the sensitivity. Irradiation to all regions leaves the body with no unexposed tissue. Under these conditions the survival values were materially reduced over those of all other types of treatment.

Quantitative Analyses of Regional Effects of X-ray Exposure

Quantitative estimates of the relative sensitivities of the different body regions were obtained by relating the survival values within the x-ray dosages for the different treatments. The basic theory was as follows.

A factor common to mice of each strain was assumed to represent the natural resistance of the strain. This factor was considered as alone responsible for the survival values attained by the unexposed control groups. It was common to mice of all groups before treatment and was in a sense the potential resistance of the strain against which the x-ray or other treatments operated to reduce viability. The factor was designated a. Irradiation to the head region contributed a factor, h, to extend or reduce life. Its value depended upon the dosage of radiation to the head but within any one dosage its effect was regarded as a constant. In the same manner irradiation effects to the mid region were regarded as due to a factor, m, and those to the rear region were considered due to a factor, r. When two regions were irradiated the effects of radiation were assumed to be additive, *i.e.*, $h + m$ for total effects of irradiation to the head-mid regions. Any unexposed cells within the body would contribute possibilities of continuing normal functions. Even a small fraction of the cells having normal functions might be of vital importance to the mouse. In consequence it was assumed that when the whole body was irradiated there were effects above and beyond those of $h + m + r$. These effects were represented by d. The full effects of whole-body irradiations were viewed as due to $h + m + r + d$. The d factor measured the importance of even a fraction of the body cells being normal in function. A system of eight equations was available for the analysis of these effects as expressed in the eight regional body treatments.

$$a = \text{value of control, unexposed mice}$$
$$a + h = \text{value of control} + \text{head exposed}$$
$$a + m = \text{value of control} + \text{mid exposed}$$
$$a + r = \text{value of control} + \text{rear exposed}$$
$$a + h + m = \text{value of control} + \text{head and mid exposed}$$
$$a + h + r = \text{value of control} + \text{head and rear exposed}$$
$$a + m + r = \text{value of control} + \text{mid and rear exposed}$$
$$a + h + m + r + d = \text{value of control} + \text{whole body exposed}$$

The values for a, h, m, r and d were obtained from the system of five simultaneous equations derived from the above by the method of least squares. These simultaneous equations are presented below. The P values in the equations were obtained as the sums of the observed values. Wherever a given factor entered into the basic equations it contributed to the corresponding P, *i.e.*, P_1 = the sum of the constants for all eight, P_2 = the sum of four equations where h entered, etc.

$$P_1 = 8\,a + 4\,h + 4\,m + 4\,r + 1\,d$$
$$P_2 = 4\,a + 4\,h + 2\,m + 2\,r + 1\,d$$
$$P_3 = 4\,a + 2\,h + 4\,m + 2\,r + 1\,d$$
$$P_4 = 4\,a + 2\,h + 2\,m + 4\,r + 1\,d$$
$$P_5 = 1\,a + 1\,h + 1\,m + 1\,r + 1\,d$$

The general solution for each of the constants was as follows:

$$a = 1/8\,(5\,P_1 - 3\,P_2 - 3\,P_3 - 3\,P_4 + 4\,P_5)$$
$$h = 1/8\,(-3\,P_1 + 5\,P_2 + 1\,P_3 + 1\,P_4 - 4\,P_5)$$
$$m = 1/8\,(-3\,P_1 + 1\,P_2 + 5\,P_3 + 1\,P_4 - 4\,P_5)$$
$$r = 1/8\,(-3\,P_1 + 1\,P_2 + 1\,P_3 + 5\,P_4 - 4\,P_5)$$
$$d = 1/2\,(1\,P_1 - 1\,P_2 - 1\,P_3 - 1\,P_4 + 4\,P_5)$$

The constants for the effects of irradiation, as measured by percentage survival, for different intensities to the different regions are shown in Table VI. The constants fitted the observations well as shown by the variations accounted for by the five factors as against the residual variation left after the fits were made.

As expected the severity of the effects of the x-rays to the different regions increased as dosage increased. The 320 r dose was not severe enough to separate the effects of exposure to particular body regions. The whole-body treatment even at this level of exposure showed the reduction in survival, d, to be greater than can be accounted for by the additive effects of $h + m + r$; d may be thought of as representing the recovery potential when any unexposed cells were present within the animal's body.

In the 480 r dose range the separation of body regions by irradiation effects was clearer. The values of m confirmed the greater sensitivity of the mid region in lowering the survival rates of the mice following x-irradiation. The head and

TABLE VI

Effects of x-irradiation to particular body regions on percentage survival of 5 different strains of mice

Constant	Dose (r)	Strains					Average
		S	Z	K	Q	Ba	
a	320	100	100	100.23	100	99.99	100.04
	480	99.55	100.25	99.54	99.53	99.54	99.68
	640	100.83	100.69	103.04	100.03	106.84	102.29
h	320	0	0	−0.68	0	0.88	0.04
	480	0.45	−0.75	0.46	0.47	1.29	0.38
	640	.01	−0.25	2.96	−0.92	−1.51	0.06
m	320	0	0	−0.68	0	−1.76	−0.49
	480	−0.45	−0.75	−0.46	−0.47	−2.13	−0.85
	640	−3.29	−2.07	−13.40	−0.04	−29.23	−9.61
r	320	0	0	0.23	0	−0.87	−0.13
	480	0.45	0.25	0.46	0.47	−0.37	0.25
	640	−2.47	−1.13	−9.91	−0.98	−14.63	−5.82
d	320	−1.75	0	−4.65	0	−8.41	−2.96
	480	−21.05	−24.00	−45.90	−34.48	−67.30	−38.55
	640	−84.37	−93.59	−78.91	−92.53	−61.46	−82.17

Variations in survival accounted for by the constants

Dose	d.f.	Mean squares				
		S	Z	K	Q	Ba
320 r	acc. 5†	15,935	16,000	15,711	16,000	15,404
	res. 3††	0	0	1.7	0	1.0
480 r	acc. 5	15,175	15,045	14,512	14,783	13,923
	res. 3	0.5	0.5	0.6	0.6	1.5
640 r	acc. 5	13,574	13,784	12,547	13,783	11,004
	res. 3	9.8	1.5	73.4	1.1	196.0

† acc. = Variation accounted for by the different regions.
†† res. = Residual or unaccounted for variation.

rear regions appeared of almost equal resistance in the strains. With the two exceptions of the *h* in the Z strain and *r* in the Ba strain, the *h* and *r* values for the other four strains showed a slightly stimulatory effect on viability. When the whole body was exposed to the 480 r x-rays the effects attributable to *d* were very severe. Since the effects as measured by the *h*, *m* and *r* values were not extreme and were quite consistent in the five strains, the order in magnitude of the *d* values for the five strains represented the levels of resistance of the strains to x-irradiation. The S mice were most resistant followed by the Z, Q, K and Ba in that order.

The effects of irradiation were more marked in all regions when the exposure

dose was 640 r. Except in the Q strain irradiation to the mid region, *m*, resulted in the severest reaction. The *r* values showed the rear region to be intermediate in resistance to irradiation. The head region as measured by *h* was least sensitive. The *d* values again portrayed the severity of exposure to x-rays in the absence of any unexposed cells or organs. The order of the strains in resistance to irradiation as observed from the *d* values with 480 r exposure and to a lesser degree with 320 r was not followed by the 640 r dose. As the *h*, *m* or *r* effects became greater the values of the *d* effects were decreased due to the limited range in which these constants operate. In the Ba strain survival from radiation had full expression from 0 per cent survival from whole-body exposure to 100 per cent survival for the unirradiated controls. The sensitivity of the mid region accounted for 29 per cent of the mortality. The sensitivity of the rear region accounted for 15 per cent more and that of the head region only 1.5 per cent. The *d* effect contributed 61 per cent additional mortality and was restricted by the 0 limit for survival.

The variation left unaccounted for after fitting the five constants was practically negligible when compared with that accounted for. The increased, but still minor, variation observed for the Ba strain at the 640 r exposure can best be attributed to the limitations of the scale for death and survival.

A like analysis of the data on length of survival (Table VII) added a little information to that already gained (Table VI). The values for *d* were more consistent with the innate resistance levels of the strains than were those based on percentage survival because the scale of measurement was not so restricted. However, at the lower x-ray doses the length of survival did not give more information than the percentage data because most mice lived the full 15 days. The mid region was again most susceptible to the x-rays with the rear next. Irradiations to the head showed little effect.

Discussion

The analyses of these data showed that sex had only inconsequential acute effects on the response of mice to irradiation. This observation agrees with that of Abrams (1951) for whole-body irradiation. Kaplan and Brown (1952) also found like reactions of the sexes in an experiment involving 1700 mice when sexes were equally distributed across several x-ray doses and fractions of the doses. Sex differences may be greater when life span effects are considered.

Comparisons of the mice without regard to strain show that the lethal effects of the whole-body x-rays increase with increase in kilovoltage, the 600 r at 250 pkv and 0.25 Cu + 1 Al filter being about as lethal as 960 r at 100 pkv Coolidge tube without filters (Gowen and Stadler, 1956).

Differences between the responses of the five strains of mice to x-irradiation were evident. Strain differentiation depended upon the x-ray dose. The more susceptible strains, Ba and K, reacted to the lower dose, 320 r, which had no effect on the survival of the S, Z and Q strains. As the exposure was increased to 480 r and 640 r the five strains were more clearly separated in their resistance levels. After exposure of the whole body to 480 r, 79 per cent of the S mice survived with a mean of 14.4 days. They were followed in order by Z, 75 per cent, 14.2 days; Q, 66 per cent, 14.2 days; K, 54 per cent, 13.4 days and Ba, 31 per cent with 12.2 days survival. The higher x-ray exposure of 640 r (250 kvp) delivered to the

TABLE VII

Effects of x-irradiation on body regions as measured by length of survival

Constant	Dose (r)	Strains					Average
		S	Z	K	Q	Ba	
a	320	15.00	15.00	15.01	15.00	15.00	15.00
	480	14.96	15.02	14.95	14.99	14.95	14.97
	640	15.06	15.06	15.24	15.01	15.64	15.20
h	320	0	0	−0.03	0.0	0.02	0.00
	480	0.03	−0.04	0.05	0.01	0.11	0.03
	640	0.01	−0.03	0.34	−0.01	−0.06	0.05
m	320	0	0	−0.03	0	−0.06	−0.02
	480	−0.03	−0.04	−0.05	−0.01	−0.18	−0.06
	640	−0.24	−0.18	−1.15	−0.06	−2.74	−0.87
r	320	0	0	0.01	0	−0.05	−0.01
	480	0.03	0.01	0.05	0.01	−0.02	0.02
	640	−0.19	−0.08	−0.84	−0.08	−1.36	−0.49
d	320	−0.21	0	−0.11	0	−0.41	−0.15
	480	−0.60	−0.73	−1.56	−0.83	−2.62	−1.27
	640	−4.03	−4.32	−4.76	−4.50	−6.04	−5.53

Variations in survival accounted for by the constants

Dose	d.f.	Mean squares				
		S	Z	K	Q	Ba
320 r	acc. 5†	360	360	359	360.0	356.
	res. 3††	0	0	0	0	.00
480 r	acc. 5	356	355	350	355	341.
	res. 3	0	0	.01	0	.01
640 r	acc. 5	333	334	312	335	277.
	res. 3	.06	.01	.61	.00	1.80

† acc. = Variation accounted for by the different regions.
†† res. = Residual or unaccounted for variation.

whole body was severe enough to appreciably narrow the range between the two extreme strains, S and Ba. The Z and Q strains interchanged positions in rank of resistance as measured by percentage survival, but were equal when the degree of severity was measured by length of survival. The strain differences were expressed only in the treatment groups where the radiation was of sufficient intensity to reduce survival of the more sensitive strains (Tables III and IV). The strains responded differently to the graded x-ray doses as shown in Table V. The interactions between x-ray doses and strains in the exposure treatment groups were about equal to the effects of the strains alone. Each strain appeared to have a reaction curve of its own.

This genetic differentiation of the strains in their response to radiation has been expanded by more recent observations on the LD50 values for 15 days under the same conditions of x-irradiation. The actual x-ray doses required to reduce survival of each strain 50 per cent were determined by exposing mice of different strains to a range of x-ray doses. The LD50 values obtained experimentally for whole body irradiations were S, 537 r; Q, 528 r; Z, 522 r; K, 481 r and Ba 438 r. From this information the Q and Z strains were more nearly alike in radiation sensitivity than was indicated in the 480 r whole-body treatment group of the experiment under discussion, but comparable to the levels determined by the 640 r exposure.

The genetic differentiation of these five strains of mice in their resistance to x-irradiation confirms the observations of Gowen and Zelle (1945) on some of these same strains and by Henshaw (1944b) on other strains. Henshaw found that C_3H mice were more sensitive than LAF_1 mice to whole-body irradiation as measured by survival and the effects on the blood picture. Kaplan and Paull (1952) showed differences between strains A and C57 black to radiation response. Differences between four strains were shown by Reinhard *et al.* (1954) with minimal lethal doses of x-irradiation. The four strains ranged in MLD values from 570 r for the Marsh strain to 492 r for C_3H. Grahn (1954), in this laboratory, observed genetic differences between six strains of mice (including S, Z and Ba) in radiation response as related to body weight changes. With respect to dosage relationships these observations are in agreement with the findings of many other investigators. Heineke (1905) reported that increased x-ray exposure reduced the efficiency of the blood-forming organs in mice. Lawrence and Tennant (1937) concluded that length of life of Swiss mice following x-irradiation was directly related to the dose given. As dose was increased they noted the increased frequency of diarrhea in the mice. Like conclusions were reported by Osborne *et al.* (1952) and by Kaplan and Brown (1952). This latter work involved adequate samples of mice for each of nine x-ray doses, 283 r to 1131 r, given in single exposures and in fractions of these total doses. From the single exposures mortality increased from 4 per cent at 283 r to 83 per cent at the 566 r dose level.

In this work the S, Z, K and Ba strains maintained the same order in both radiation response and in subsequent response to mouse typhoid. This experiment introduces a new strain, Q. The Q strain showed a difference in its reactions. It was quite resistant to x-rays but extremely susceptible to mouse typhoid. Q mice did not fit in the observed pattern of the other mice with respect to the two responses. The leucocyte level for the Q strain has not been determined but is of real interest. The level of the white blood count of this strain would be indicative of the causal relationship of leucocytes to disease resistance (Weir *et al.*, 1953; Gowen and Calhoun, 1943) or to radiation sensitivity (Gowen, 1952). The K strain, not included in the six strains previously tested, followed in line with the other strains in both disease resistance and numbers of leucocytes (Thompson, 1952).

Differences in regional sensitivities to x-rays became evident when the body regions and combinations thereof were irradiated. The order of increasing sensitivity was head, rear, head-rear, mid, head-mid, mid-rear and head-mid-rear or whole-body. The dosages were not adequate to appreciably affect survival when the head, rear or head-rear were x-rayed. Only those four groups in which the

mid third was involved gave noticeable differences for the strains and dosages. Comparison of the mid-rear to the head-mid indicated a greater radiation sensitivity of the rear third as contrasted with the anterior third of the body. The reduction in survival and length of survival was between four and five times as severe following exposure of the head-mid as it was following that of the mid alone. The mid-rear reaction was around 16 times as severe as that of the mid alone. The increased reduction was affected by the particular regions rather than by the proportion of the body exposed.

Although no attempt was made in this investigation to associate specific organs to radiation sensitivity, specific organs or tissues were implicated by their inclusion within a given region as shown in Plate 1. The posterior third of the body implied the intestines, testes, bladder, etc., whereas the mid portion included the spleen, liver, etc. Our observations on the sensitivity of the mid-rear region in part confirmed those of Warren and Whipple (1922). They found in dogs that the abdomens, comparable to the rear plus a good portion of the mid region in these data, were more sensitive to x-irradiation than the head and thoracic region. They attributed this increase in mortality from x-rays to severe toxemia and septicemia enhanced by exposure of the intestines. Bond *et al.* (1954) arrived at similar conclusions. Chrom (1935) separated the effects of exposure to the rear region from those to the rest of the abdomen. He concluded that the rear region, including the intestines, was not as sensitive as the upper part of the abdomen. These data also supported the conclusion of Osborne *et al.* (1952) that bacteremia and intestinal damage were not closely correlated. The observations of these investigators were supported by those which have been derived from our studies.

The greater sensitivity of the mid region as shown in our data may be related to the response of the lymphoid tissues, spleen and nodes. Heineke's (1905) observations, supported by those of Läwen (1909), showed the blood and blood-forming tissues to react strongly to x-radiation in rats, rabbits, mice and guinea pigs. Further emphasis on the hematopoietic tissues in radiation response as well as in recovery comes from the investigations of Lawrence and Tennant (1937), Ellinger (1945), Henshaw (1944a, 1944b, 1944c), Bloom and Jacobson (1948) and Barrow and Tullis (1952) to name but a few. Again the data of this paper may be interpreted as indicating the significance of the proper functioning of these organs as they affect survival.

The important role of the spleen, a center of hematopoiesis, has been demonstrated by the increased survival obtained by shielding this organ from x-radiation (Jacobson, 1954; Wissler *et al.*, 1953; and Bond *et al.*, 1950) and by the partial protection afforded irradiated animals by injections of splenic or bone marrow tissue homogenates (Jacobson, 1954; Cole and Ellis, 1953; Lorenz *et al.*, 1952; and Barnes and Loutit, 1955). Again the behavior of these organs under irradiation has parallel significance to the data on x-ray survival presented in this paper.

The quantitative interpretation of these data is, however, somewhat different. The authors cited above have tended to consider each organ studied as all-important to irradiation survival. Our data show that while the different regions were significant, their effects on survival were less impressive than these other investigators suggested.

The fitting of constants to the regional body effects offers a new approach to the evaluation of radiation effects to different regions of the body. This quantitative

estimation of regional effects confirms our previous observations that exposure of the head region or anterior third of the body is less effective in reducing survival than either of the other regions. X-ray doses in the range used did not appear to influence this minor effect. Exposure of the rear region was shown to be detrimental to survival at the higher exposure level of 640 r. This effect was consistent in the five strains although of minor importance to the Q strain. Irradiation to the mid region showed the greatest consistent reduction in survival for all strains. Increase in dosage increased the severity of the reaction in the strains. Greater mortality was observed in the more susceptible strains, Ba and K. Only the Q mice showed a different reaction; exposures of the rear or head were nearly as detrimental as those to the mid region, although the effects of the three regions were small.

The greatest reduction in survival resulted from whole-body irradiation. The amount of this reduction as measured by the constant, *d*, was above that due to the combined effects of head, mid, and rear exposures. The *d* values represent that percentage of the total mortality that resulted when all cells of the body were exposed and is in addition to the mortality resulting from the combined exposures to the three portions of the body. The magnitudes of these *d* values particularly after 640 r, but also after 480 r, indicate the importance of at least some unexposed cells in facilitating the recovery of the irradiated animal. The large differences between the combined effects of the three regions as compared to the body irradiation, *d*, suggest that any unexposed cells contribute materially to the protection of the mouse from irradiation. This was also indicated by the work of Gershon-Cohen *et al.* (1951), who showed that shielding areas comparable to 15 per cent of the total body area of mice resulted in reduced mortality from radiation. Almost equal protection was afforded the mice by shielding the liver, or lung or abdomen. They concluded that viability of the animal was increased by shielding any part of the hematopoietic system and was not confined to special organs. Jacobson *et al.* (1951) compared the hematopoietic recovery in mice irradiated with regions or organs shielded from exposure to 1025 r. Shielding of the spleen gave the greatest increase in survival as well as in hematopoietic recovery. Shielding of the liver lobe or portion of the intestines increased survival, but to a lesser degree. These results were associated with only somewhat less recovery of hematopoiesis. However, shielding of the head gave nearly the same increase in survival as that of the intestine, but recovery in blood formation was only partial. The protection of the right hind limb, but not of the kidney, was also beneficial to survival. The results from the head and intestine shielding point to the influence of cells, tissues or systems, other than those involved in hematopoiesis, as contributing to the radiation response. Although injections of suspensions of splenic tissues contributed most, bone marrow and liver tissues as well as body tissues of embryos contributed to recovery of the irradiated host (Jacobson *et al.*, 1955). Our own observations do not rule out a major role for the hematopoietic system, but the increased severity of radiation to the whole body over that to the regions suggests that any cells, regardless of their apparent morphological specificity, could stimulate recovery. The scattered radiation absorbed in the shielded regions would tend to increase the effects to the exposed regions. Consequently the presence of scattered radiation would tend to decrease the *d* value as obtained here (Table VI).

In all cases the Ba mice show the largest effects of the regional exposures; the

d values have been minimized, however, by the limitation of 0 per cent survival. The K strain as previously shown is next in susceptibility to radiation followed by the Q, Z and S strains. The resistance of the S strain appeared related to the resistance of its cells to better withstand radiation even though exposures to the mid and rear regions were more detrimental to survival of the S mice than to the Z or Q mice. The resistances of the Z and Q strains appeared to be determined by the interactions of all cells and regions. The increased susceptibility of the K and Ba strains was contributed to by the proportionately greater sensitivity of the mid and rear regions. The five strains, however, showed quite similar reactions but to different degrees in their responses to x-irradiation. These results were in contrast to those of Reinhard *et al.* (1954). They found marked differences in four strains of mice in radiation sensitivity of the head as compared to that of the remainder of the body. Kaplan and Paull (1952) also showed strain differences between A and C57 black mice in the results of spleen shielding. Protection of the spleen was more important to A mice than to C_3H mice. This observation of the A strain was in accord with that of Lorenz *et al.* (1952). They observed differences between four strains of mice in their responses to the protection afforded by bone marrow cell suspensions. Intravenous and intraperitoneal injections of the homogenate increased survival for two strains, L and LAF_1. For strains A and C_3H intraperitoneal injections were of little value in decreasing mortality.

The data indicate the importance of maintaining at least some cells free of irradiation if the organism is to survive. The body cells retain a significant totipotency which contributes to maintaining the organism as a whole even though the cells may have differentiated to extreme types anatomically or physiologically.

Summary and Conclusions

1. The influence of x-irradiation absorbed in three body regions and in the combinations of these regions has been measured by three subsequent responses: survival to radiation, natural resistance to disease and ability to acquire resistance following contact with the disease agent, *S. typhimurium*. The effects of irradiation are presented in this paper. Papers on natural and acquired resistance will follow. The experiment was designed as a factorial with five genetically differentiated strains of mice, S, Z, K, Q and Ba; four levels of radiation: 0 r, 320 r, 480 r and 640 r; eight treatment groups and two sexes. All mice were 46 ± 3 days of age when irradiated from a 250 pkv x-ray source operated at 30 ma with 0.25 mm. Cu + 1 mm. Al filter at a dose rate averaging 170 r/minute. For the initial treatment the strains and sexes were well balanced, at least 50 mice in each of the 25 different treatment groups. The bodies of the mice were marked off in three regions, head *h*, mid *m*, and rear *r*, each comprising one-third of the body length. These regions, their combinations and their controls with each irradiation account for the 25 treatment groups. Shielding was done with $\frac{1}{8}$-inch lead. As most deaths occur between 7 and 12 days, an interval of 15 days was allowed for expression of any direct effects due to radiation. Deaths were recorded daily.

2. Percentage survival and length of survival were the two measurements used for determining the reactions in each response.

3. The sexes responded in like manner to x-irradiation. A penetration or wavelength effect was indicated in these data. The reactions of the mice to the whole

body irradiation at 250 pkv, 0.25 Cu + 1 Al filter 600 r were similar to those for 100 pkv, Coolidge tube, no filtration, 960 r.

4. Within the x-ray dose range used the responses of the strains to x-irradiation were shown to be partially genetically determined.

5. The levels of radiation resistance were in the order from resistant to sensitive: S, Z, Q, K and Ba. After 480 r total-body exposure the survival percentages of the five strains were: S, 79; Z, 75; Q, 66; K, 54 and Ba, 31. This order does not coincide with the order known to be followed in natural resistance to mouse typhoid: S, Z, K, Q and Ba.

6. Shielding of one-third of the body protected the mice of the five strains from 320 r and 480 r x-radiation, and to much lesser degree, depending upon regional exposures, from 640 r. The dose of 640 r was not of sufficient intensity to allow full expression of strain differences for the different regional exposures.

7. Whole-body exposure to 320 r reduced the 15-day survival for the more sensitive strains Ba and K, 480 r decreased survival in the five strains; 640 r was severe enough to largely overcome the genetic differences between the strains.

8. The mid region of the mouse was most sensitive of the three single regions, and more sensitive than the combined head and rear regions. The radiation effects were determined by the region rather than by the area of the body exposed.

9. The mid region in combination with the rear region showed greater sensitivity than the head-mid region. All strains were reduced in survival by exposure of the mid-rear to 640 r, whereas only the less resistant strains Ba and K showed the effects from the exposures of the less sensitive regions.

10. The decrease in survival showed the mid region as most sensitive for S, Z, K and Ba, followed by the rear portion with the anterior third of the body resistant. These four strains responded in the same manner but to different degrees.

11. The reactions of the Q strain separated it from the four other strains. Its level of radiation resistance with respect to the other strains was in contrast to its low level of natural resistance to mouse typhoid. Radiation resistance and natural resistance to this disease have been found highly correlated in seven of our strains of mice. The Q mice show comparable though slight sensitivity to x-radiation in the three body regions. Mortality in the Q strain was largely confined to whole-body exposures of 480 r and 640 r. This suggests that the Q mice have no particular center of radiation sensitivity, but that mortality is the result of the interactions of the cells throughout the body.

12. The data on length of survival confirmed the results from percentage survival and contributed additional information for those reactions that resulted in 0 or near 0 per cent survival.

13. The lead shielding, ⅛-inch in thickness, was adequate to protect the given regions from radiation. The three groups completely shielded when exposed to the three dosages of x-rays did not quite duplicate the 0 r group in their reactions. Mortality appeared unrelated to the x-ray dose, as 100 per cent survived 640 r, 98.4 per cent the 480 r and 99.6 per cent the 320 r.

14. The strains exhibited their own characteristic responses to different x-ray doses as was evidenced by the large values for dosage × strain interactions. These interactions were real, representing the expressions of genetic resistance and as such would contribute to the strain effects.

15. The effects of the relative sensitivities of the body regions were estimated quantitatively as well as qualitatively. The quantitative estimates compared favorably with the qualitative observations.

16. The additivity of the regional effects is supported by the little unaccounted-for variation remaining after fitting the constants derived on the assumption that *h*, *m*, *r* and *d* were additive in effect.

17. Mortality from whole-body irradiation was only partially accounted for by the combined mortalities resulting from the exposures to the different regions of the body. The effect of total-body exposure over and beyond that of the combined regional effects, *d*, was interpreted as a measure of the reaction when all cells of the body of the mouse had been exposed, or when all recovery potential had been affected.

18. The whole-body effect, *d*, was large and suggested that all cells may contribute to recovery regardless of the organ or system involved. As a consequence, protection of any cells of the body during exposure to radiant energy may stimulate recovery.

19. In terms of host resistance the unexposed cells over-compensate. The extreme over-compensation initiated by cells in different unexposed regions when cells of other regions are inactivated points to the significance of all body cells in resistance whatever their degree of tissue or organ differentiation.

20. These results indicate that the body cells retained a totipotency to assist in maintaining the organism as a whole despite the differentiation which these cells may have undergone since their stem cells left the embryologically differentiating primitive tract. They further show the importance of maintaining at least a small portion of the body free from irradiation if irradiation exposure should occur through accident or calculated risk.

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SEXUAL DIFFERENTIATION IN THE TELEOST FISH XIPHOPHORUS HELLERII, AS MODIFIED BY EXPERIMENTAL TREATMENT

HENRY H. VALLOWE

Department of Zoology, Ohio University, Athens, Ohio

Attempts to alter the pattern of sexual differentiation in the Mexican swordtail fish, *Xiphoporus hellerii,* show a stability in sexual development usually not credited to this species. The swordtail has long been used as a classic example of lability of sexual differentiation and determination. The widespread reference to sex-reversal occurring in the species suggested a need for further investigation.

This work was aided by the guidance and kind supervision given by the late Dr. Carl R. Moore of the University of Chicago. I am indebted to Dr. Myron Gordon for his generosity in supplying the fishes used in this study. It is a pleasure to acknowledge the cooperation of the Schering Corporation which supplied the hormone preparations used.

Animals and Their Treatment

The fishes used were descendants of ten pairs from the Genetics Laboratory of the New York Zoological Society. They were reared under conditions similar to those described in earlier papers (Vallowe, 1952, 1953). The original ten pairs produced 618 males and 490 females in a period of approximately three years. The presence of a modified anal fin was the criterion used to establish maleness. Fish not possessing the gonopodium were anesthetized in Chlorobutanol and examined before a strong light. The characteristic amber color of the ova apparent in the ventral region of the posterior end of the body cavity was the criterion used to classify fish as females.

The sex ratios which have been established for this species show wide variations. Witchi (1939) considers *X. hellerii* to be a species without any hereditary sex determining mechanism although the presence of such mechanisms has been shown for other species in the genus. Geiser (1924) presents a comprehensive table showing the sex ratios reported by various authors. Harms (1926) gives a ratio of 24 males to 35 females. Bellamy (1922) reports 100 males to 66.7 females. Friess (1933) also reports a high proportion of males and considers temperature an important influence on the sex ratio. Breider (1935) made a study of the effects of light, nutrition, space and water and found that these had no certain influence on the sex ratio. In light of many conflicting reports, one cannot assume that a preponderance of one sex is due to sex-reversal unless all factors which may influence sexual differentiation are duly considered.

As a basis for interpreting the experimental results reported, a large series of fish were killed at various stages of development, beginning two weeks before birth

and continuing up to more than two years of age. The gonads of these fish were examined to determine the normal pattern of sexual development and differentiation. As early as three days after birth, the gonads could be recognized as potential testes or ovaries by the relative size, number and arrangement of the primordial germ cells. In the young male the germ cells were slightly smaller than in the female. In addition, there were fewer germ cells in each gonad primordium and they were concentrated at the periphery of the gland. In the young female the slightly larger cells were in greater numbers and were well distributed. The embryonic development of this species closely parallels the description given by Goodrich *et al.* (1934) and Dildine (1933, 1936) for *L. reticulatus,* that by Wolf (1931) for *X. maculatus,* and those by Geiser (1924) and Medlen (1950) for two species of *Gambusia.* Although sexual differentiation is conspicuous in these species shortly before birth, Essenberg (1923), Bailey (1933) and Regnier (1938) have shown it to be somewhat delayed in *X. hellerii.*

The differences which distinguish the early testes and ovaries are very subtle characteristics. The appearance of the early ovary and the indifferent gonad are so similar that Regnier (1938) considers that all the fish are born female and that the males later undergo a sex change. This introduces an unnecessary complication which is easily resolved if one assumes a longer period of indifferent development. Once differentiation begins, it progresses in an orderly sequence of events. Chavin and Gordon (1951) describe the differentiation of the testes in *X. maculatus* by using a series of six stages. With only slight modifications, these stages were used in the present study to classify the testes of *X. hellerii.* Gordon and Aronowitz (1951) make the observation that the histological structures of the testes of adult *X. maculatus* and *X. hellerii* are practically identical. The present study has shown that the development of the testis and the pattern of the differentiation of its structures are strikingly similar in the two species. Except for the time spent in the late stages of immaturity, the sequence of events is identical.

The process of sexual differentiation in *X. hellerii* as described by Essenberg (1923) and Van Oordt (1925) traces the origin of the definitive sperms to the epithelial cells of the testis tubules. Wolf (1931), Goodrich *et al.* (1934), and Chavin and Gordon (1951) were unable to find comparable stages of transformation in the species which they studied. The testis in *X. hellerii* is the acinus-type characteristic of the vivaparous Cyprinodonts. The arrangement and development of the structures within the testis indicate that the acini form from pre-existing acini at the periphery of the testis. As the acini are formed, they are pushed along the tubules toward the central ducts by the growth of new acini at the periphery. The peritoneal covering of the testis, the stroma cells, and the epithelium of the ducts and tubules gave no evidence of transforming into germ cells.

It is slightly more difficult to distinguish the newly differentiated ovary from the indifferent gonad because the arrangement of the cells remains about the same. However, the relative size and number of the cells characterize the gonad as an immature ovary. Early in the differentiation process the primordial germ cells are found scattered throughout the stroma of the ovary. When the fusion of the paired ovaries is completed, the germ cells come to lie within the wall of the cavity formed between them or in the stroma layer which supports this wall. In the mature female oogonia are found developing within the wall of the cavity, hence the name germinal epithelium. The larger and more advanced cells are pushed

outward toward the peritoneal covering. Essenberg (1923) feels that all the primordial germ cells disintegrate and do not take part in the formation of the germinal epithelium. Although there is great difficulty in determining the origin of the cells within the germinal epithelium, no disintegrating primordial cells were encountered in the early stages of gonad fusion and cavity formation. The primordial cells appeared to remain in normal active condition and were arranged in small groups surrounded by stroma cells. In the germinal epithelium it is possible to see all stages of transition in the shape of the nuclei from elongate oval, typical of the epithelial cells, to the spherical nuclei of oogonia. A comparable situation exists in *L. reticulatus* according to Goodrich *et al.* (1934) and leads them to the conclusion that epithelial cells as well as primordial germ cells may give rise to the definitive ova. Wolf (1931) arrived at a similar conclusion. The origin of the sex cells appears to be different in the two sexes of these species. While the primordial germ cells are the only source of definitive sperms, the peritoneal cells forming the wall of the ovarian cavity may contribute to the formation of definitive ova.

The following series of experiments reports the effects of estrogenic and androgenic hormones on gonad development. The results of the treatments described are interpreted in light of their effect on the normal sexual differentiation.

Experiments and Results

Immature fish

Young sexually immature fish (49 to 55 days old) were given nine weekly injections of 0.01 cc. of sesame oil containing 0.25 mg. of testosterone propionate into the body cavity. During this period of time there was a conspicuous thickening and elongation of the anal fin rays, a growth and pigmentation of the sword-like extension of the caudal fin and an intensification of the lateral line and dorsal fin coloration. In short, the young fish appeared to be miniatures of the sexually mature adult males. The histological picture presented by the gonads of these fish was one of radical change in the immature ovaries and one of general stimulus in the young testes.

The ovaries of the testosterone-injected immature females lacked any sign of ova. There were follicles present but these were filled with a loose collection of cells. This indicates that ova had been present previously, but that resorption had taken place. In other follicles primary spermatocytes, spermatids and spermatophores were observed. The ovarian cavity was obscured and much stroma filled the gonad. The brood-mate control females showed ovaries in which fats and oils were being deposited in the maturing ova.

The testes of immature males treated with this androgen showed an acceleration in spermatogenesis. The tubules and sperm ducts were filled with all stages of spermatogenesis including well formed spermatophores. The epithelium lining the sperm ducts was greatly hypertrophied but otherwise the testes appeared very similar to those of mature males. Brood-mate control males showed testes in less advanced stages of development.

Another group of immature fish at comparable ages was given nine weekly injections of 0.01 cc. sesame oil containing 0.00083 mg. of estradiol benzoate. At the end of the treatment period these fish were deep-bodied, showed only faint

indications of coloration and, in general, appeared to be miniatures of the adult females. The histological picture presented by these fish was the reverse of the situation found in those injected with testosterone. The ovaries of the estradiol-treated females showed a general stimulus while the testes of the treated males showed radical changes.

The testes of the estrogen-treated males showed a modification from the bipartite gonad to a fused structure in which the two lobes were no longer distinct. The peritoneal covering of the gonad had thickened, the germinal elements were no longer concentrated at the periphery of the gonad, the sperm ducts were obscured, and the blood vessels were enlarged. Some acini contained what appeared to be oogonia. Other acini contained disintegrating cells which resembled primary spermatocytes. The testes were larger than those of the brood-mate controls. In some of the gonads the sperm ducts appeared only as spaces with no organized epithelium. Two new dorso-lateral cavities had formed and were lined with a well organized epithelium. Oocytes were common in the testes showing this degree of modification.

Ovaries of the estradiol-injected females showed ova in the stage of oil deposition. There was a slight increase in the amount of stroma present but the blood vessels and follicular epithelium appeared normal. The germinal epithelium appeared very active and abundant oogenesis was observed.

Mature fish

The histological picture presented by the testes of sexually mature males after ten weekly injections of 0.02 cc. sesame oil containing 0.00166 mg. estradiol benzoate was one of general suppression and destruction. The most conspicuous effects obtained were those of an enlargement of the sperm ducts and the destruction of the spermatogenic elements. In normal mature males the sperm ducts were paired, centrally located tubes which contained spermatophores suspended in a lightly staining, non-granular fluid. In estrogen-treated males the ducts were greatly enlarged and in some cases occupied most of the testes when viewed in cross-section. They seemed to be distended with fluid and contained spermatophores in various stages of disintegration. Quantities of free spermatozoa were observed in the lumen of the ducts; this condition is not found in normal mature males. However, free spermatozoa are found in the ducts of senile males which have passed the reproductive age and are no longer capable of fertilization.

The effect on the germinal elements was most drastic in the intermediate stages of spermatogenesis. While spermatozoa and spermatogonia were still abundant, primary and secondary spermatocytes and spermatids were usually reduced in number or entirely absent. The acini which, by their position, should have contained the intermediate stages of spermatogenesis, had hypertrophied walls in which the cell outlines were conspicuous. These acini were smaller than normal and were either filled with disintegrating germ cells or were completely empty. The reduction of the acini was accompanied by a slight increase in the amount of stroma tissue and the appearance of a fibrous tissue network. The blood vessels were numerous and enlarged. The testes appeared less bipartite than the normal condition, but none were found in which fusion was as complete as that found in the ovary.

The histological picture presented by the ovaries of females given ten weekly injections of 0.02 cc. sesame oil containing 0.5 mg. testosterone propionate was also one of general suppression and destruction. None of the ovaries showed any signs of spermatogenesis even when injections were continued for as long as eighteen weeks. A conspicuous activity was noted in the germinal epithelium but there was no indication that the primary germ cells being proliferated could be spermatogonia. The cells were in groups of as many as ten, but their arrangement, size, and staining properties were strikingly similar to the oogonia found in normal ovarian development. The larger ova that were present in the gonad were in various stages of disintegration. Only a few of the ovaries examined showed the presence of mature ova; in most cases, there remained only large, empty follicles which were in various stages of collapse. There seemed to be little effect of the hormone treatment upon the smaller ova; they were still firm, were surrounded by a well organized follicle, and were in their normal arrangement in the ovary. There was very little increase in the amount of stroma in the ovary and only isolated areas in which a fibrous network was formed. On the other hand, the blood vessels had increased in size and were found throughout the ovarian tissue. In gross appearance, the ovaries of the androgen-treated females resembled those of immature normal females.

In the empty follicles and ovarian cavity of many of the virgin ovaries the presence of a secretion was observed. This secretion appeared as a mass of crumpled membranes which is normally found only in the non-virgin ovary. The frequency of its appearance indicated that this is a typical response to androgenic injections and it may be indicative of an expulsion of mature ova from their follicles. However, no mature eggs were found in the ovarian cavities or oviducts. If the eggs were completely evacuated from the body, they were never found in the aquaria.

The ovaries of the gravid females did not differ from the ovaries of virgin females given the same treatment. In two cases where the females were killed three weeks after the initial injection of testosterone propionate, embryos in early stages of development still remained in the follicles. The characteristic membrane-like secretions were observed in the follicles of some of the gravid females.

Discussion

The results obtained from the study of the normal sexual differentiation of *X. hellerii*, and from the attempts to alter the pattern of this differentiation by experimental treatment, point to a stability in sexual development and differentiation which has usually not been credited to this species. Much of the experimental and descriptive work dealing with Xiphophorin fishes has emphasized the ease with which the secondary sexual characteristics of these species can be made to respond to hormonal treatment. The effects of many hormone substances on the primary sex organs have also been described but the results are not always in agreement. However, in all cases of sex-reversal reported for adult *X. hellerii* the change has always been from female to male, whether such reversals were naturally occurring phenomena or whether they were induced by hormone treatments. During the course of this investigation no case of natural sex-reversal was observed in the laboratory population; further, the sex ratio obtained for this

population indicates that unobserved sex-reversals could not have occurred in large numbers. The adult sex ratio and the sex distribution of the fishes used in the study of the normal development of the gonads did not indicate that a shift in the ratio occurred between the juvenile and mature populations. Essenberg (1923) postulated that a sex-reversal of 50 per cent of the immature females would explain the shift he observed in the sex ratio (a change from a ratio among immature fish of three females to one male to the adult condition of one female to three males).

A possible explanation of naturally occurring sex-reversal in adult fish, in which virgin females become males, lies in improper initial classification. In this study only those fish which possessed amber ova that could be observed through the body wall when viewed against a strong light source were designated as females. This procedure reduced to a minimum the possibility of erroneous sex classification. The classification of immature males as females may account for a few, but certainly not all, of the cases of hormone-induced sex-reversal reported by other investigators.

Another factor which may play a significant role in the discrepancies of the results obtained from experiments with *X. hellerii* lies with the variations in the fish used. The very important reviews by Gordon (1931, 1937) concerning the history of Xiphophorin species as aquarium fishes give substantial evidence to indicate that the *X. hellerii* available from commercial hatcheries have possibly been produced by hybridization with *X. maculatus*. For the aquarist the hybrid fish has many traits that are desirable. The hybrid is usually more highly colored, more robust and larger than either parent species, and more prolific in the production of large broods of young. These factors would account for its selection and propagation by commercial hatcheries. Gordon and Rosen (1951) suggest that the hybrid possesses an imbalanced chromosomal arrangement that may have endocrinological significance in that this imbalance may initiate abnormal or nonfunctional gonads in the hybrid. Although hybrids may usually be identified by color or color patterns, some are practically identical with the wild-type swordtail. The few cases of natural sex-reversal that have come to my attention have always been in hybrid fishes or in fish with unknown origins. A strong possibility exists that the sex-reversals reported by Essenberg (1926) occurred in hybrid stocks. (He gives the origin of the stock as the Crescent Fish Farm, a commercial fish hatchery.) Further application of this possibility suggests that the work of other investigators may also be based on commercially available hybrid strains rather than on pure species stocks.

The results of the experimental treatments given to the pure species used in this study were found to be in close accord with the results obtained by many investigators using the closely related species which are not known for their tendencies to produce sex-inversions. The response to the techniques employed indicated that these fish were in no way aberrant but were in close agreement with the other members of the viviparous Cyprinodont group.

Although the immature fish developed the germ cells of the opposite sex under hormone treatment, the sexually mature fish did not when subjected to the same hormone preparations. Since the gonads are homologous structures, it is possible that they retain a few common properties until differentiation becomes so advanced that these must be sacrificed. Evidence from the treatment of maturing males

with estradiol benzoate shows that the ability of the testis to produce ova is retained from a short time after gonopodium elongation. However, this response is lost after the later stage of male development, *i.e.*, gonopodium differentiation, is initiated. In the mature male the effects of estrogenic treatment were a destruction and suppression of existing elements. Unlike the results obtained in immature and maturing males, there was no stimulus to develop female characteristics.

Immature females produced spermatozoa within their ovaries under the influence of testosterone propionate. This response, however, was not found in the ovaries of mature females given twice the amount of the same hormone preparation.

Summary

1. The normal sexual development and differentiation of *Xiphophorus hellerii* have been observed in the light of the adult sex ratio and the differentiation of the gonads from birth to maturity. The sexual differentiation has been shown to follow a definite pattern in both sexes. The gonads of both sexes are homologous and indifferent at birth, but within a few days testes and ovaries can be distinguished by the relative size, number and arrangement of the primordial germ cells. No atypical gonads were discovered in the course of this study which would indicate the possibility of sex-reversal.

2. Injections of testosterone propionate induced spermatogenesis in the ovaries, of immature females but had no comparable effect in mature specimens.

3. Injections of estradiol benzoate induced oogenesis in the testes of immature males but had no comparable effect in mature specimens.

4. Improper initial sex classification and hybrid origin of the fish used are suggested as possible explanations for some of the discrepancies between the results obtained in this study and those reported in earlier investigations.

5. The results of the study of the normal sexual development and the attempts to alter the differentiation pattern by experimental means indicate a stable sexual development and differentiation for this species.

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